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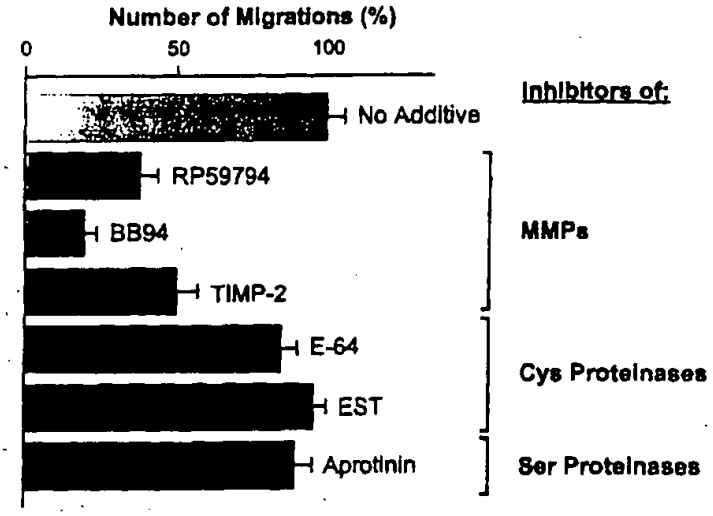

(57) Abstract

Bone metabolic disease is treated by inhibition of the production or action of membrane-type matrix metalloproteinase (MT-MMP) or the matrix metalloproteinase 12 (MMP-12) involved in the resorptive activity of osteoclasts. Inhibitors for MT-MMP and MMP-12 and membrane-associated metalloproteinase activity include peptides and analogues of peptides generated using a PEGA bead library, antisense nucleic acid agents and antibodies. The proteinases MT1-MMP and MMP-12 are found to be expressed in osteoclasts and may be selectively inhibited.

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THE USE OF PROTEINASE INHIBITORS FOR PREVENTION OR REDUCTION OF BONE RESORPTION

The present invention relates to the reduction of the rate of bone resorption by targeting the action or production of proteases.

Human bone is constantly undergoing remodelling. The fine balance between bone formation and bone resorption is regulated by local and systemic factors and by physical forces acting on various cells including, in the bone environment, osteoblasts and osteoclasts. However, in several bone metabolic diseases including most importantly osteoporosis and osteolytic bone metastasis, the balance is disturbed resulting in a sustained pathological net bone resorption.

Osteoporosis is a systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a subsequent increase in bone fragility and susceptibility to fracture. Post-menopausal osteoporosis is a chronic disease which affects millions of women throughout the world and it has an enormous economical and social impact on society.

Reduction of bone resorption is believed to be an appropriate way to prevent and treat several metabolic bone diseases, including osteoporosis and osteolytic bone metastasis. Agents such as steroid hormones (especially oestrogen), calcitonin and bisphosphonates are able to suppress bone resorption and have been used for prevention and treatment of osteoporosis and/or osteolytic bone metastasis. However, these therapeutic agents fail to achieve satisfactory effects in some cases, due to subject limitation or uncertain efficacy. There is therefore need of a new prophylactic/ therapeutic method for preventing or treating accentuated bone resorption.

Removal of the mineralised osseous substance, i.e. organic matrix embedded in deposits of calcium phosphate

salts, is a complicated process. Though still a controversial subject, it seems probable that osteoclasts are the only cells capable of bone resorption. The progressing bone loss in patients with osteoporosis is caused by an increase in the activity of osteoclasts.

The expected life cycle of osteoclasts involve the following major phases:

1. recruitment of haematopoietic stem cells, the early precursor of osteoclasts,
2. proliferation and differentiation,
3. fusion into multinuclearity,
4. attachment to the resorptive bone surface,
5. polarisation and removal of mineralised osseous substance, and
6. death by apoptosis, necrosis or a more random process.

These phases are, however, not necessarily separate events, thus, e.g. differentiation might take place during migration to the resorptive surface and fusion might take place on the bone surface. All these phases represent possibilities for intervention in order to regulate the level of bone resorption.

Traditionally, proteolytic enzymes have been known to play a role in degradation of the organic matrix of bone. The knowledge about proteolytic enzymes involved in bone resorption mainly comes from *in vitro* and *in vivo* studies of the effects of natural and particularly synthetic enzyme inhibitors. Furthermore, histochemical and immunocytochemical characterisation of enzymes in bone cells and tissues as well as more recently identification of enzyme-encoding mRNA in osteoclasts and other bone cells has increased the information about proteolytic enzymes involved in bone resorption. The proteolytic enzymes of major relevance to osteoclastic bone resorption seem to be members of the families of cysteine proteinases and matrix metalloproteinases (MMPs).

The use of proteinase inhibitors in disease control has been suggested in several scientific publications and in patents and patent applications. For MMP inhibitors the main focus has been the potential of inhibitors in treatment of cancer and tumour metastasis, but also diseases such as arthritis, ulcers, periodontal and bone diseases, HIV infection, corneal and other eye diseases, diabetes and myocardial infarction have been the target of these speculations and ensuing early experiments (reviewed by Birkedal-Hansen et al, 1993 [2]).

In some particular cases, however, the studies have been emphatic leading to particularly important conclusions and products of relevance to the use of proteinase inhibitors in disease control. Selected peptidyl derivatives were shown to be effective inhibitors of metalloproteinases reaching K_i-values down to 5 pM for MMP-2 by kinetic studies based on a fluorogenic synthetic peptide substrate incubated with MMP-1, -2 or -3 and the substances were orally active and non-toxic in mice at suitable doses (WO94/25434).

Membrane-type matrix metalloproteinases (MT-MMPs) were originally identified in cancer cells and have been implicated with the migration of these cells (Sato *et al* 1994[13]). Based on this disclosure, its seems that the use of MT-MMP inhibitors will be appropriate for the reduction of the spread of tumours. No studies have, however, yet described inhibitors of MT-MMPs and thus no data are available on the use of MT-MMP inhibitors as agents in the treatment of diseases. From the usually low selectivity of synthetic MMP-inhibitors, it seems probable that some established MMP-inhibitors will inhibit MT-MMPs. Furthermore, cDNA encoding MT1-MMP (also referred to in the literature as MT-MMP-1 and as MMP-14) as well as anti-MT1-MMP antibodies have been suggested, though rather unspecifically, as useful for application not only in the diagnostic area but also in other medical fields (EP-A-0685557 and WO95/25171).

The inhibition of cathepsins is considered another possible way of reducing bone resorption by using proteinase inhibitors. Several cathepsins are produced by osteoclasts and though still somewhat controversial, they are apparently involved in the degradation of organic matrix in the acidic environment of the sub-osteoclastic resorption zone. Recently a novel cathepsin named cathepsin K, cathepsin O or OC2 was cloned from osteoclasts and osteoclast-like cells by several independent groups. It was suggested that development of antisense probes or synthetic inhibitors to this proteinase could be of value in the treatment of several diseases including osteoporosis. For cathepsin L several compounds have been produced for use as specific inhibitors in the treatment and prevention of osteoporosis (EP-A-0611756).

The general use of hybrid molecules for conferring specificity to cell- and tissue-interacting agents has been proposed in several modifications including hybrids consisting of three parts including not only a cell-binding ligand and a chemical entity to be introduced into the target cell but also an intermediate part constituting a translocation domain for enabling the entrance of the chemical entity into the cell (WO91/0987). Another approach to resist clearance and degradation and ease the uptake in cells of peptides and proteinase inhibitors is by administering them as lipid conjugates (WO93/01828).

Speculations about the biological roles of osteoclastic proteinases have been almost entirely focused on their potential ability as mediators of degradation of organic bone matrix in the sub-osteoclastic resorptive zone. However, our recent findings have shown that proteolytic enzymes are also very important for the migration and attachment of osteoclasts to the resorptive surface (Blavier & Delaissé, 1995[3]). Furthermore, the proteinase-dependent migration of immature osteoclasts seems to be associated with the maturation into active bone-resorbing

osteoclasts as well as of importance for the events leading to fusion into multi-nuclearity, i.e. osteoclast differentiation processes.

Being an earlier phase of the osteoclast life cycle, interference by an inhibitor of a proteolytic enzyme involved in osteoclast migration and/or attachment might be more effective than inhibition of an enzyme involved directly in the resorptive process. This type of interference will also be easier to accomplish since the secreted enzymes of the migrating cells are not protected from inhibition as they are when secreted into the tightly sealed resorption zone which is formed when the active polarised osteoclasts attach to bone.

We have now discovered that an MT-MMP closely related to or identical to MT1-MMP, previously identified in cancer cells not related to bone, is expressed by osteoclasts. It may be expected that this osteoclast MT1-MMP plays an important role in the action of osteoclasts, probably being implicated in their migration to their site of action at which to degrade bone (see Examples 1, 2, 3-2 and 3-3 and Figures 1 to 3). This finding indicates that also other membrane-associated metalloproteinases such as other MT-MMPs or members belonging to families of non-matrix type of membrane metalloproteinases (e.g. meltrins and "A disintegrin and metalloproteinase"'s, ADAMs)) could be produced by osteoclasts.

Furthermore, we have identified and characterised the full length gene and the encoded protein of osteoclast metalloelastase MMP-12, a proteinase hitherto believed to be almost specifically expressed in macrophages, where it is obligatory for the invasion of these cells through basement membranes. Since macrophages and osteoclasts are closely related cell types both originating from the haematopoietic stem cell and differentiating late in its development, a similar role of MMP-12 in osteoclast invasion and migration is likely (see Example 3-4 and Figures 4 to 6).

The present invention provides the use of an agent in the manufacture of a medicament for the treatment of bone metabolic disease, characterised in that the agent acts by inhibition of the production or action of a membrane associated protease or the matrix metalloprotease MMP-12 involved in the resorptive activity of osteoclasts. More preferably, the invention provides the use of an agent in the manufacture of a medicament for the treatment of bone metabolic disease by inhibition of the production or action of a metalloproteinase involved in the resorptive activity of osteoclasts. Particularly, inhibition of the production or action of an MT-MMP but also of other membrane-associated metalloproteinases such as a meltrin or an ADAM as well as a secreted MMP such as MMP-12.

The treatment may be for prevention or for cure of such diseases.

Preferably, the metalloproteinase is involved in the recruitment, proliferation, differentiation, or migration of osteoclast precursor cells or in the migration, fusion, attachment, polarisation, activity in removal of mineralised osseous substance, or death of osteoclasts.

Though MT-MMP and MMP-12 produced by osteoclasts and osteoclast precursors is a major target for the inhibitory agent of the invention, the invention also includes regulation of bone metabolism by inhibition of non-osteoclastic proteinases which influences the life cycle of osteoclasts. Other bone cells such as osteoblasts and chondrocytes are able to produce both latent and active forms of MMPs, cathepsins and plasminogen activator as well as natural inhibitors of some of these enzymes. These enzymes might be important for the initial degradation of the bone surface exposing the underlying mineralised matrix to subsequent osteoclastic action (Delaissé & Vaes 1992[5]) and they might be involved in the degradation of collagen fibres either released from the bone by the action of osteoclasts or still remaining in the resorption pit after the osteoclast has left (Foged *et al*, 1996[6]). Furthermore, latent pro-forms of osteoblastic enzymes stored in bone

might be activated during osteoclast resorption. Finally, proteolytic enzymes of non-osteoclastic origin might have a chemotactic role in regulating the migration and maturation of osteoclasts.

The agent may be selectively inhibitory of MT1-MMP or MT-MMPs broadly, of MMP-12 or MMPs broadly, or of membrane-associated metalloproteinases or metalloproteinases broadly.

The agent may be an antibody selectively immunoreactive with an MT-MMP. Such an agent may alternatively be an antisense oligo-nucleotide or oligo-nucleotide analogue directed against a gene involved in the production of an MT-MMP or an agent regulating MT-MMP activity. It may be an MT-MMP substrate mimic inhibitor. It may be a broad spectrum matrix metalloproteinase (MMP) inhibitor or a broad spectrum membrane-associated metalloproteinase inhibitor. It may also be a peptide, peptide analogue or other peptide mimicking agent obtained by screening an appropriate library for compounds reactive with an MT-MMP, an MMP or a membrane-associated metalloproteinase.

A preferred inhibitor provided by the invention is the peptide S-K-Y-P-J-A-L-F-F-K (SEQ ID No.1) (J being the single letter code of hydroxyproline) and inhibitory variants thereof such as the peptide analogue S-K-Y(NO_2)-P-J-A-L-F-F-K(Abz) (SEQ ID No.2).

In an alternative aspect, the invention includes the use of an agent in the manufacture of a medicament for the treatment of bone metabolic disease by inhibition of the recruitment, proliferation, differentiation, or migration of osteoclast precursor cells or in the migration, fusion, attachment, polarisation, or death of osteoclasts. Preferably, said agent produces said inhibition by inhibiting the production or action of a proteinase.

The invention includes an anti-bone resporption agent comprising a proteinase inhibitor active against a proteinase involved in bone resorption operatively linked to a ligand having binding specificity targeting the inhibitor to said proteinase or to the environment of the proteinase.

The invention includes a new protease termed rabbit osteoclast MT1-MMP having the amino acid sequence given in Figure 1 and Figure 2, as well as an isolated nucleic acid coding for such a protein, e.g. one having the sequence set out in Figure 1. Proteins having high e.g. more than 75% eg more than 90% or 96% homology to the said rabbit osteoclast MT1-MMP are included also, as is human osteoclast MT1-MMP and isolated nucleic acid sequences encoding it.

The invention also includes a new protease termed rabbit osteoclast MMP-12 having the amino acid sequence given in Figure 4 and Figure 5, as well as an isolated nucleic acid coding for such a protein, e.g. one having the sequence set out in Figure 4. Human osteoclast MMP-12 and isolated nucleic acid sequences encoding it as well as other proteins and nucleic acid sequences with a high homology (e.g. at least 50%, preferably at least 70, 80 or 90%) to rabbit osteoclast MMP-12 are also included in the invention.

Inhibition of proteolytic activity can be obtained in several ways and by several classes of agents. The inhibition could be direct, i.e. by an agent acting directly either on the proteinase in its active form(s) inhibiting its proteolytic activity or substrate recognition or on the latent form of the proteinase inhibiting its conversion into active proteinase. The most relevant directly acting inhibitors of proteinases include:

1. natural inhibitors which form specific complexes with an active proteinase and in some cases even with its latent pro-enzyme (e.g. tissue inhibitors of metalloproteinases, TIMPs);
2. antibodies or antibody fragments which e.g. neutralise the active site or block the substrate recognition site;
3. synthetic pseudo-substrates which specifically interact at the catalytic site (e.g. synthetic peptides linked to a chelating group) or the natural substrate recognition site; and

4. so-called entrapping reagents which are cleavable substrates which when cleaved undergo a conformational change which leads to entrapment of the proteinase (e.g. a-macroglobulins).

The inhibition, however, could also be indirect i.e. by an agent regulating either the expression and/or production of the proteinase (e.g. a natural transcription factor or its naturally regulating systemic or local factor, or a synthetic antisense probe specifically binding to and blocking the mRNA encoding the proteinase) or by an agent influencing the level or activity of a natural regulator of the proteinase (e.g. an inhibitor of an enzyme responsible for catalytic activation of the target proteinase).

The development of many types of proteinase inhibitor is assisted by having the proteinase itself available. The production of proteinases may be performed either directly in cultures of isolated osteoclasts or indirectly by transfection of an expression plasmid containing proteinase encoding cDNA into a recipient cell line. For proteinase production in osteoclasts, the majority of e.g. MMP-9 is produced in its latent proform (pro-MMP-9) and therefore needs a subsequent activation process if the active form is required. The amount of proteinase obtained from production in osteoclast is severely restricted by:

a) the non-proliferative nature of osteoclasts in culture and
b) the technical difficulties in isolation of native osteoclasts in high numbers and purity.

For illustration, the production, purification and activation of osteoclastic pro-MMP-9 is described in Example 3-1. In contrast, both latent and active proteinase can be produced directly by recombinant techniques depending on whether the expression plasmid-transfected into the recipient cell is designed to contain the complete cDNA or a cDNA devoid of the region encoding the propeptide moiety of

the latent enzyme. Since active proteinases are generally less stable than their corresponding latent pro-enzymes and particularly under cell culture conditions might be degraded, production of latent proteinases is often preferable. For illustration, the identification and cloning of cDNA encoding several osteoclastic MMPs or parts thereof, including MMP-9, MMP-12 and MT1-MMP is described in Examples 1, 2 and 3-4.

Apart from natural regulators of metalloproteinase and particularly MMP production and activity, agents inhibiting metalloproteinases (including MMPs and especially MT-MMPs and MMP-12) involved in one or more phases of the osteoclast life cycle can include:

1. a substance which interacts at a specific site of the metalloproteinase or MMP thereby reducing its proteolytic activity to recognise a natural substrate, e.g. anti-MMP antibodies and fragments thereof as well as synthetic, peptide-mimicking proteinase inhibitors;
2. substances which influence the transcription or translation of metalloproteinase or MMP;
3. substances stimulating the level or activity of a natural inhibitor of metalloproteinase or MMP; and
4. substances reducing the level or activity of a natural activator of metalloproteinase or MMP, e.g. a substance analogous to the description in 1. and 2. but regulating a proteolytic enzyme responsible for activation of latent MMP.

Examples 5 and 6 below describe the development of inhibitory agents; the production and use of anti-proteinase antibodies (Example 5); the production, identification and characterisation of synthetic, peptide-mimicking proteinase inhibitors (Example 6 a-e); and the design and use of antisense probes to proteinase mRNA (Example 6f).

Anti-proteinase antibodies are central tools for the development of proteinase inhibitors and under appropriate conditions can be used as inhibitors themselves (see Example

5e and Figure 9). Thus, the applications for anti-proteinase antibodies and parts thereof are several and in particular anti-MMP antibodies and antibody fragments will be useful:

1. In the production of recombinant MMP by use in immuno-blotting or a similar immunodetection method for identification of clones expressing recombinant proteinases.
2. In affinity chromatographical purification of native or recombinant MMPs by immobilisation on activated resins produced for affinity columns such as e.g. divinyl sulfone agarose.
3. In immunoassays such as ELISA or RIA for quantitative determination of the specific MMP concentration in samples for diagnostic analysis e.g. tissue extracts, sera or urine samples, and in samples for research analysis e.g. cell culture medium.
4. In immunocytochemical identification of MMP-expression on the protein level by incubation with bone cells or tissue sections. As shown in Example 5, this can also lead to a demonstration of a particular cellular localisation of a MMP and thereby aid in the clarification of its biological role.
5. In the characterisation of MMP-activity by use as specific inhibitory agents. Antibodies have shown the highest specificity as MMP inhibitors in test tubes (i.e. selectivity for a particular MMP and not others) and therefore will be important tools for characterisation of individual proteinases (Birkedal-Hansen et al, 1993[2]). Especially, antibodies raised by immunisation with peptides mimicking a region comprising the catalytic site of a particular MMP could be expected to interfere with the proteolytic activity of this member but not other members of the MMP-family and thereby be of importance for the demonstration of the specific role of particular proteinase in bone metabolism.

6. In the manufacture of a medicament for the treatment of bone metabolic disease by use as direct MMP inhibitors or as constituents of hybrid MMP inhibitors. Two general principles for using anti-MMP antibodies or fragments thereof for treatment of bone metabolic disease are relevant: as direct inhibitors of proteinase activity or as site-directing agents merely assuring that another inhibitory agent is transported to the right target cell or tissue, e.g. by hybridisation on the protein or gene level of the antibody or a fragment thereof to a peptide-mimicking synthetic inhibitor. In both cases the use of antibodies in treatment of a bone metabolic disease requires its administration to animal or man in a proper pharmaceutical composition to avoid degradation and to ensure a beneficial effect.

Synthetic peptide and peptide-mimicking inhibitors of proteinases are promising agents for use for treatment of bone metabolic disease by inhibition of the action of proteinases involved in the recruitment, proliferation, differentiation, or migration or osteoclast precursor cells or in the migration, fusion, attachment, polarisation, removal of mineralised osseous substance, or death of osteoclasts. Several methods for production of peptide and peptide mimicking inhibitory agents are available, two of which are described in Example 6 (a-e).

One is based on a recently developed beaded polyethylene glycol cross-linked polyamide (PEGA) resin designed for peptide synthesis and with an open structure permitting biologically active proteins into the interior (Meldal *et al*[11], 1994: Meldal & Svendsen, 1995[12]). The PEGA bead peptide library was developed for the complete characterisation of the specificity of proteinases in general and can be used for identification of first synthetic peptide substrates of osteoclast proteinases and subsequently inhibitors after a well-functioning substrate has been identified. In the first step of this procedure

millions of randomly synthesised fluorogenic peptides are screened for their ability to become hydrolysed during incubation with an osteoclast proteinase. The major purpose of this step is to identify a synthetic peptide substrate suitable for use in the second step of the procedure, i.e. the identification of inhibitors of the same proteinase. However, the identification of substrates might lead directly to inhibitory agents, since substrates with high affinity for the proteinase but little ability to become hydrolysed (i.e. pseudo-substrates) can act as reversible inhibitors. In Example 6b, we report the finding of a peptide-mimicking molecule (CL-1) identified by incubation of MMP-9 with a PEGA bead substrate library, which has a low K_m (3.4 μM) but also a low k_{cat}/K_m (<500 $M^{-1}s^{-1}$) suggesting its potential use as an inhibitor of osteoclastic MMP-9. Even better inhibitory characteristics of pseudo-substrates can be expected after modification of the originally identified substrates, e.g. either by linking peptide-mimicking substrates to chelating groups such as hydroxamates, thiols, phosphonamidates, phosphinates and phosphoramidates (reviewed by Birkedal-Hansen *et al*, 1993[2]) or by designing pseudo-substrates which easily forms acyl-proteinase complexes but which hydrolyse slowly due to interaction with the binding site on the enzyme for the leaving group (Baggio *et al* 1996[1]).

In the more regular cases where the identification of an appropriate synthetic substrate (i.e. showing a low K_m and a high K_{cat}/K_m by incubation with the proteinase) is obtained either by the first step of the PEGA bead procedure or by simply being already commonly available, synthetic peptide inhibitors can be identified among millions of randomly designed peptides in a PEGA bead synthetic peptide inhibitor library (Meldal and Svendsen, 1995[12]; Meldal *et al*, 1997[21]). The screening is based on the rare ability of some peptides to inhibit the hydrolysis of the established synthetic peptide-mimicking substrate. Inhibitors of MMPs, MT-MMPs and membrane-associated metalloproteinases can be found by this method also.

A novel modification of the original PEGA bead inhibitor technology was developed in order to optimise the synthesis of MMP inhibitors. It has previously been shown (Galardy et al, 1992[19]) that substituting the cleavable peptide bond (-CO-NH-) in a peptide substrate of fibroblast collagenase by a phosphorus-containing bond e.g., a phosphinate ($-PO_2-CH_2-$), phosphonamidate ($-PO_2-NH-$) or phosphonate ($-PO_2-O-$) bond can cause inhibition of the proteolytic activity. For the first time, this knowledge has been used in combination with the PEGA bead technology by extending the group of building blocks used for synthesis of putative inhibitory peptide analogues on the PEGA-beads from just natural amino acids (including hydroxyproline) and their corresponding D-forms to also including pseudo dipeptides such as $NH_2-P1^P/^CP1'-COOH$, $NH_2-P1^P/^NP1'-COOH$ or $NH_2-P1^P/^OP1'-COOH$; where the two normal amino acids (P1 and P1') instead of being linked through the peptide bond are linked through the phosphinate, phosphonamidate or phosphonate bond ($^P/_C$, $^P/_N$ or $^P/_O$). This allows the synthesis of random PEGA-bead inhibitor libraries with a structure such as: X1-X2-$P1^P/^CP1'$-X3-X4-"linker"-PEGA, where X1 to X4 are natural amino acids and $P1^P/^CP1$ is a phosphinate pseudo dipeptide (as described in Example 6c and Figures 12-15).

By employment of the PEGA-bead substrate library technology, it has been possible to identify peptide sequences which are of use in the design of novel highly specific MMP-substrates (see Example 6 a and b). These substrates facilitate the design and use of PEGA-bead inhibitor libraries both through the use of one of these selective substrates in the library and through the use of the substrate sequence data for the design of the structure of the randomised inhibitors in the library (Meldal and Svendsen, 1995[12]; Meldal *et al*, 1997[21]). Particularly in the design of PEGA-bead inhibitor libraries based on inhibitors with a phosphorous containing bond, the substrate data were used for determination of the two amino acid R-groups around the phosphinate, phosphonamidate or phosphonate of the pseudo dipeptide (see Example 6c). Furthermore, the design

of selective inhibitors based on the characteristics of the novel MMP-substrate specificities will be facilitated (see data for CL-1, CL-21, CL-25 and CL-29 in Example 6b). Finally, the specific substrates could become important tools for selective detection and quantification of MMPs in tissue samples in diagnosis and research.

The other method for identification of peptide and peptide mimicking inhibitory agents is based on the use of positional combinatorial peptide inhibitor libraries. A few members of these libraries of randomly synthesised peptides having in a single amino acid position an abnormal amino acid, such as a D-amino acid instead of an L-amino acid, in some case will act in an inhibitory way to a particular enzyme, probably due to a pseudo-substrate effect. If an inhibitory signal is obtained by incubation of a positional combinatorial peptide inhibitor library with a proteinase or a biological model system including essential proteinase activity, the peptide(s) in the library responsible for this inhibition must be subsequently identified by systematic segmentation of the library as described in Example 6 (d-e) for incubation of positional combinatorial peptide inhibitor libraries with murine foetal metatarsal cultures. Some preferred inhibitory libraries and peptide structures provided by the invention are the libraries X-X-w-X-X, X-X-l-X-X and X-X-w-Y-X and the peptides C-L-w-Y-L, C-L-w-Y-M, C-Y-w-Y-L, V-Y-w-Y-M and L-F-w-Y-L, where X are natural amino acids including hydroxyproline, and w and l are D-tryptophan and D-leucine, respectively (see Example 6e).

Comparing the two methods, the major advantage and disadvantage of the PEGA bead library are the immediate identification of inhibitors and the need for incubation with a preferably purified proteinase preparation in a test tube, respectively. The major advantage and disadvantage of the positional combinatorial peptide inhibitor library is the possibility to screen directly for an inhibitory effect in a biological test system and the need for several cumbersome segmentations of the initial library to identify the agent originally causing the inhibition, respectively.

Finally, one feature of the positional combinatorial peptide inhibitor library can be seen as both favourable and non-favourable, since the functional background for an inhibitory response induced in the biological system by this type of library is uncertain i.e. the inhibitory peptides might not be proteinase inhibitors but have other regulatory functions.

A review by Eggleston and Mutter of methods for producing inhibitors mimicking inhibiting peptides appears in "Chemistry in Britain" May 1996, pages 39-41[18]. The techniques reviewed may be applied to peptides identified by the methods discussed above.

The benefits of using antisense probes to proteinases can be divided into two major aspects, an early aspect and a later aspect. The antisense probes are important tools for evaluation of the role of the corresponding proteinase in a biological process, because they can be used at an early stage of a study when anything else than the oligonucleotide sequence of this proteinase is unknown, and this even with usually high specificities i.e. with only a minor risk of cross-reaction to other proteinases if the design of the antisense probe and the experimental conditions are appropriate. Antisense probes were used successfully for inhibition of MMP synthesis by fibroblasts (Lin et al, 1995[9]), and interfered with the proton pump activity of osteoclasts when assessed in both cell and tissue cultures (Laitala and Vaananen, 1994[8]). Another major aspect of using antisense probes is their possible application in the treatment of diseases caused by over-expression of particular genes. For specific reduction of proteinase levels, gene therapeutic use of antisense probes to MMPs may be expected to be effective.

The identification of an antibody-derived or synthetic peptide-mimicking inhibitor of an osteoclast proteinase may be followed by appropriate modification of this compound to assure its use as a medicament for the treatment of bone metabolic disease. Several characteristics are necessary, particularly sufficient uptake and stability in the living

organism to assure a beneficial effect, sufficient tissue or cell specific action to assure maximal effects at the target site of the organism relative to effects at non-target sites including acceptable levels of side effects, and a pharmacologically acceptable dose- and time-response to the treatment.

Administration of proteins, peptides and peptide-like substances to animals and humans requires protective routes of administration and/or protective formulation of the peptide in order to avoid degradation of the compound. Though protective encapsulation for oral administration of peptides and peptide-like agents is a technology currently undergoing significant improvement, stabilisation of the agent itself prior to administration is advantageous. For peptide-mimicking MMP-inhibitors this has been possible by chemical modification of an initially identified compound apparently without important changes in its inhibitory capacity (Brown & Giovazzi, 1995[4] and P. D. Brown personal communications June 1996).

Targeting of a proteinase inhibitor to e.g. osteoclasts and osteoclast precursors, can be obtained by two general means. One, is if the inhibitor due to its intrinsic specificity selectively reacts with the proteinase present on these cells either because the proteinase at this target cell is particularly available to the inhibitor (due to e.g. the localisation of the cell, the localisation of the proteinase in the cell or simply by a local high concentration of the proteinase) or because the proteinase when produced by these cells is different from the corresponding proteinase as it is expressed in other cells and tissues (due to e.g. post-translational modifications). The other way to obtain a specificity is by making hybrid molecules or conjugates combining one part of the agent having proteinase-inhibitory characteristics with another part having antibody or ligand specificity for the particular cells or tissue. These hybrids can be made by recombinant expression of fusion-proteins after cloning of a hybrid cDNA. E.g. a piece of cDNA encoding the osteoclast-

specific ligand calcitonin (or a receptor-binding part thereof) can be ligated to another piece of cDNA encoding a peptide inhibitor for an osteoclast proteinase. Hybrids can also be conjugates of two compounds e.g. by chemically linking an amino-bisphosphonate, which has high affinity for hydroxyapatite in bone, or an antibody specific for a component exposed in the osteoclast membrane, such as the calcitonin receptor with a peptide or peptide-mimicking proteinase inhibitor.

The invention will be further described and illustrated with reference to the examples which follow and the appended drawings in which:

Figure 1 shows the nucleotide (SEQ ID No.3) and deduced amino acid sequence (SEQ ID No.4) of the MT1-MMP or MT1-MMP analogue identified in rabbit osteoclasts;

Figure 2 shows a comparison between the amino acid sequence of the novel MT-MMP identified in rabbit osteoclasts (Rabbit) (SEQ ID No.4) and the previously reported amino acid sequences of Human (SEQ ID No.5), Rat (SEQ ID No.6) and Mouse MT1-MMP (SEQ ID No. 7). Positions with an amino acid identical in all 4 proteins are indicated (*);

Figure 3 shows schematically the structure of three MT1-MMP cDNA constructs and the corresponding control construct used in Example 3-2;

Figure 4 shows the nucleotide (SEQ ID No.8) and deduced amino acid sequence (SEQ ID No.9) of the MMP-12 or MMP-12 analogue identified in rabbit osteoclasts;

Figure 5 shows a comparison between the amino acid sequence of the novel MMP-12 identified in rabbit osteoclasts (Rabbit) (SEQ ID No.9) and the previously reported amino acid sequences of Human (SEQ ID No.10), Rat (SEQ ID No. 11) and Mouse MMP-12 (SEQ ID No.12). Positions with an amino acid identical in all 4 proteins are indicated (*);

Figure 6 shows schematically the structure of a MMP-12 cDNA construct and the corresponding control construct used in Example 3-4;

Figure 7 shows the effect of various proteinase inhibitors on the migration of purified osteoclasts through collagen coated membranes. The values are relative to the number of migrations observed in the absence of proteinase inhibitor.

Figure 8 shows the effect of an MMP-inhibitor on pit formation by purified osteoclast seeded on dentine slices which were either not coated or coated with collagen. The values are relative to pit formation in the absence of collagen coating and MMP-inhibitor;

Figure 9 shows the dose dependent inhibitory effect on MMP-9 proteolytic activity of sera from mice immunised with the conjugated femta-peptide RSGAPVDQMFPGVPL (SEQ ID No.13) (peptide B, mimicking a region of the rabbit MMP-9 hemopexin domain) alone or together with purified intact rabbit osteoclast pro-MMP-9. No inhibitory effect was observed for sera from non-immunised mice and for mice immunised with another non-related femta-peptide (peptide A). The values are relative to the average relative fluorescence generated during 30 minutes of incubation of the synthetic quenched fluorogenic substrate Mca-PLGL-Dpa-AR-NH_2 (Bachem) (SEQ ID No. 14) with a pre-incubated mixture of purified activated MMP-9 and the appropriate dilutions of 9 different control sera (non-immunised or immunised with non-relevant femta-peptides);

Figure 10 shows the relationship between the initial velocity of enzymatic hydrolysis and the substrate concentration determined by continuous fluorometric assay of MMP-9 or subtilisin with either (A) MR2: Abz-G-P-L-G-L-L^{nor}-A-R-Y(NO_2)NH_2) (SEQ ID No.15) or (B) CL1: Abz-S-K-Y-P-J-A-L-F-Y(NO_2)-D (SEQ ID No.16). Assays were

performed at 37°C, pH 7.5 and fluorescence read at λex = 320 nm and λem = 425 nm. Peptide origin and kinetic parameters are reported in Table 1;

Figure 11 shows inhibition of hydrolysis of CL1 by the MMP-inhibitor RP59794, but not the cysteine proteinase inhibitor E-64. MMP-9 (80 pmol) or subtilisin (3.4 pmol) were pre-incubated with either RP59794 or E-64 in a total volume of 40 µl for 5 min at 37°C. Subsequently, 1 ml of 2.8 µM CL-1 was added and the incubation continued for 2 to 70 hrs. Inhibitor is listed in final concentrations;

Figure 12 shows the synthesis of the phosphinate analogue to hydroxyproline for use as a building block in the subsequent generation of a hydroxyproline-methionine phosphinate pseudo dipeptide (see also Figure 13). The phosphinic acid analogue to trans-hydroxyproline is synthesised from potassium D- or L-erythronate. After bromination at the 2 and 4 position the acid is transformed into the methyl ester by methanol quenching. The 2-position is reduced and the ester converted into the alcohol by sodium borohydride reduction. The primary alcohol is oxidized by sodium hypochlorite to the aldehyde and condensed with tritylamine. The imine formed is reacted with bis-trimethylsilyloxyphosphine to yield the phosphinate. Upon acid hydrolysis and intra-molecular substitution of the bromine the free hydroxy-proline is obtained;

Figure 13 shows the synthesis of the hydroxyproline-methionine phosphinate pseudo dipeptide for use in preparation of the PEGA bead phosphinate inhibitor library IIa (see Example 6c). The phosphinic acid analogue of hydroxyproline (see Figure 12) is derivatised with benzyloxycarbonyl chloride. 2-methylene-4-methyl mercapto-butanoic acid ethyl ester was synthesised from diethylmalonate sodiation and reaction with methyl mercaptoethyl chloride followed by selective basic ester hydrolysis, acid decarboxylation and reaction with

formaldehyde in the presence of piperidine. These reactions can be performed on a large scale. Reaction with the phosphinic acid analogue of hydroxyproline gives the dipeptide isosteric phosphinate. The phosphinate is protected by reaction with adamantylbromide followed by ester hydrolysis with sodium hydroxide. The Cbz group is cleaved hydrogenolytically and the free amine protected by reaction with FmocCl and sodium carbonate;

Figure 14 shows the synthesis of the glycine-leucine phosphinate pseudo dipeptide for use in preparation of the PEGA bead phosphinate inhibitor library IIb (see Example 6c). The phosphinic acid analogue of glycine is synthesised from tritylamine and formaldehyde to give the imine which is reacted with bis-trimethylsilyloxyphosphine obtained from ammoniumphosphinate and hexamethyl disilazane. The product is deprotected by acid hydrolysis and is derivatised with benzyloxycarbonyl chloride. 2-Methylene-4-methyl pentanoic acid ethyl ester was synthesised from diethylmalonate sodiation and reaction with isobutylbromide followed by selective basic ester hydrolysis, acid decarboxylation and reaction with formaldehyde in the presence of piperidine. Reaction with the phosphinic acid analogue of glycine gives the dipeptide isosteric phosphinate. The phosphinate is protected by reaction with adamantylbromide followed by ester hydrolysis with sodium hydroxide. The Cbz group is cleaved hydrogenolytically and the free amine protected by reaction with FmocCl and sodium carbonate;

Figure 15 shows the development and structure of the PEGA bead phosphinate inhibitor library (IIa) based on the hydroxyproline-methionine phosphinate pseudo dipeptide. The invariable quenched fluorescent substrate (here: Ac-Y(NO_2)PLJMKGK(Abz)G-"Linker"-) (SEQ ID No.17) and the randomly variable phosphinate inhibitor (here: $X_1X_2J^P/^CMX_3X_4$-"Linker"-) are independently associated to the PEGA bead. Alternatively an FmocLys(Aloc) residue can be used to

obtain orthogonal protection and incorporation of the two compounds and the order of synthesis of the library and the substrate may be reversed. This gives the possibility to use the same library with several substrates. The analogous library (IIb) was prepared similarly by using an invariable substrate corresponding to MR1 (see Table 3) and a randomly variable phosphinate inhibitor $X_1X_2G^P/^CLX_3X_4$-"Linker"-;

Figure 16 shows inhibition of the $^{45}Ca^{2+}$-release from foetal murine metatarsals cultured for 4 days in the presence of positional combinatorial pentapeptide inhibitor libraries. The results for 5 selected libraries with the sequence X-X-D-X-X are shown. In these 5 cases D was either D-isoleucine, D-leucine, D-lysine, D-serine or D-tryptophan, and X were randomly varying L-amino acids. In contrast to the libraries with D-lys and D-ser, the pentapeptide libraries with a D-ile, D-leu or D-trp at the third position induced a significant reduction of bone resorption. The MMP-inhibitor RP59794 was included as a positive control.

Example 1

Isolation of cDNA encoding fragments of osteoclastic proteinases.

The use in PCR of degenerate nucleotide primer sets (designed from existing data describing the amino acid sequences of proteinases) for cloning of osteoclastic proteinases was exemplified by the studies described below leading to the identification of MMP-9, MMP-12 and MT1-MMP mRNA in rabbit osteoclasts:

a. Isolation and purification of osteoclasts

Osteoclasts were isolated from 10-day-old rabbits (125-150 g) according to a method described previously (Tezuka *et al*, 1992[15]) but with some modifications. Briefly, bone cells were released from marrow-depleted long bones and shoulder blades by mincing and mechanical agitation. A preparation

of unfractionated bone cells rich in osteoclasts was isolated by centrifugation (30 x g, 5 min) and seeded into tissue culture dishes. After a settling period of 90 minutes, non-adhering cells were removed, and cultivation continued for 20 hrs at 37°C and 5 to 7.5% CO_2 in *a*-MEM (pH 7.3) supplemented with 5% foetal calf serum. The cells were washed with PBS and then treated with 0.001% pronase E and 0.02% EDTA for approximately 10 min. to release all non-osteoclastic cells. The purified osteoclasts were cultured for another 2 hrs before isolation of mRNA.

b. Amplification of MMP cDNA fragments by PCR, molecular cloning and homology analyses

To identify possible MMP gene expression by rabbit osteoclasts, cDNA reverse-transcribed from mRNA from the purified osteoclasts was subjected to PCR with degenerate primers designed from conserved regions of MMP genes. Briefly, the poly(A)$^+$RNA from purified osteoclasts was prepared using a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden); single strand cDNA was synthesised from mRNA by use of a cDNA synthesis kit (Pharmacia); and aliquots of the synthesised cDNA were amplified by PCR with degenerate primers corresponding to the conserved amino-acid sequences in either the cysteine switch region (PRCGVPD (SEQ ID No.18)) or the region resembling a cleavage site for furin (RRKRYA (SEQ ID No.19)) in combination with the catalytic domain (GDXHFDXXE (SEQ ID No.20), where X is a variable amino acid) present in most members of the MMP-family. The PCR reactions were cycled 45 times through the following steps: 1 min at 94°C, 1 at 55°C, 1 min at 74°C. Three cDNA bands 330-340, 380-390 bp and 560-570 bp in length were identified by electrophoresis in a 1% agarose gel. The cDNAs were purified and cloned into a pCRII vector (Invitrogen, San Diego, CA) according to the instruction manual and subsequently characterised by nucleotide sequencing.

The high expression of MMP-9 mRNA by rabbit osteoclasts is well-known and from previous characterisation of the MMP-9 gene the expected size of MMP-9 cDNA fragments amplified with degenerate primers used in this PCR would be 336 bp. Our cloning and subsequent nucleotide sequencing confirmed that the isolated 330-340 bp cDNA originated from MMP-9.

The cloning of isolated 560-570 bp cDNA, resulted in a clone, B4 with a length of 567 bp which by nucleotide sequencing was found to share more than 80% similarity with a segment of the human metalloelastase (MMP-12) gene. The presence of mRNA encoding MMP-12 has previously been preliminarily identified in rabbit osteoclasts by partially sequencing randomly chosen cDNAs of an osteoclast cDNA library (Sakai *et al*, 1995[12]) (see also Example 3-4).

The cloning of isolated 380-390 bp cDNA, resulted in another clone, A3 with a length of 387 bp, which shared more than 90% similarity with the human MT1-MMP cDNA sequence previously reported in cancer cells (Sato *et al*, 1994[13]). Since neither MT-MMPs nor any other membrane-associated proteinases have been previously identified in osteoclasts, the remaining part of this example as well as Example 2 describes studies of A3 and MT-MMP in osteoclasts.

c. *Isolation of MT1-MMP cDNA from an osteoclast cDNA library*

A rabbit cDNA library (Tezuka *et al*, 1994[15]) was screened by colony hybridisation, using the random-primed 32P-labelled PCR product of A3 as a probe. By screening $1x10^5$ clones, one positive clone was identified and made into the plasmid form according to the instruction manual (Stratagene, lambda ZAP vector). This positive clone contained a cDNA insert of 1,842 bp which was isolated and sequenced. An open reading frame consisting of 1716 bp initiated with an ATG codon at nucleotide position 127 was found. According to gene bank searches, an identical nucleotide sequence did not exist and the highest similarity was 91% to the human MT1-MMP gene. Figure 1 shows the nucleotide sequence of the cloned insert. The deduced

amino-acid sequence of the insert showed 96% similarity with human MT1-MMP (Figure 2). There were no additions or deletions of specific sequences when compared to MT1-MMP of other species. Based on further comparisons of amino acid sequences of other MMPs, we concluded that the isolated novel cDNA encoded the rabbit homologue of MT1-MMP or of a closely related but previously unreported human osteoclast MT-MMP.

d. Nucleotide sequence analysis

The nucleotide sequence analysis of the A3 PCR fragment and of the rabbit MT1-MMP cDNA clone from the cDNA library was determined from both strands by the dideoxy chain-termination method using the Qiagen-purified plasmid DNA (Qiagen, USA), the Sequenase kit (U.S.B., USA), and either pBluescript SK primers (Stratagene, USA) or synthetic oligonucleotide primers.

Example 2

Identification of MT1-MMP in osteoclasts.

The novel identification of MT1-MMP in osteoclasts was further substantiated by the studies described in the following examples:

a. Cells and organs for RNA preparation

Brain, kidney, liver, lung, calvaria, spleen and alveolar macrophages were isolated from 10-day-old rabbit. Bone stromal cells were obtained from a culture of unfractionated rabbit bone cells (Tezuka *et al*, 1992[15]) in alpha-MEM containing 10% FBS until confluence, and then subcultured 4 times. In all cases total RNA was prepared as reported previously (Tezuka *et al*, 1992[15]).

b. Northern blotting

To investigate the mRNA expression of MT1-MMP in purified osteoclasts and to compare its level with that in

other tissues and cells, we performed Northern blotting. Five micrograms of total RNA isolated from various organs and cells were blotted on nylon membranes after formaldehyde agarose gel electrophoresis, and hybridised with radioactive probes. The A3 PCR fragment and a fragment of human MT1-MMP cDNA (position 1647-2880, Sato et al, 1994[13]) as well as (for quantitative normalisation) a synthetic oligonucleotide corresponding to 28 S ribosomal RNA were used as probes. The cDNA probes were radiolabelled with a multiprime DNA labelling system (Amersham International plc., Buckinghamshire, England) using [alpha-^{32}P]dCTP and the oligonucleotide probe was radiolabelled with a 5'-end labelling kit (Amersham) using [gamma-^{32}P]ATP. Hybridisation was performed as described previously (Tezuka *et al*, 1992[15]) and visualised by a Phosphorimager SF (Stratagene, La Jolla, CA). For both MT1-MMP probes, we found the same pattern of distribution as those reported previously for adult human tissues (Takino et al, 1995[14]; Will and Hinzmann, 1995[17]), and in addition a prominent expression of MT1-MMP in purified osteoclasts. It was noteworthy that expression was not detectable in liver and brain and low expressions were found in bone stromal cells and alveolar macrophages.

c. In situ hybridisation

The expression of MT1-MMP in osteoclasts in vivo was examined by *in situ* hybridisation on sections of rabbit metatarsals. Consecutive paraffin sections of metacarpal bones of new-born rabbits were prepared as previously described (Blavier and Delaissé, 1995[3]). A fragment of rabbit MT1-MMP cDNA (position 1-318, corresponding to 126 nucleotides in the non-coding 5'-region and 192 in the region encoding the N-terminal part of MT1-MMP) was used for probe synthesis. Digoxygenin-labelled antisense or sense RNA probes were prepared by use of a DIG RNA labelling kit (Boehringer Mannheim) according to the instruction manual and compared to paraffin sections stained for tartrate-resistant acid phosphatase (Blavier and Delaissé, 1995[3]). Many tartrate-resistant acid phosphatase-positive multi-

nucleated cells were positive for MT1-MMP, whether they were attached to calcified cartilage or to bone.

d. Immunocytochemistry

An important property of the MT1-MMP in previous investigated non-osteoclastic cells is its localisation in their plasma membrane. The expression of MT1-MMP at the protein level and its cellular localisation in osteoclasts was investigated by immunocytochemistry. Unfractionated rabbit bone cells were seeded on glass coverslips. After 1.5 hr cultivation the non-adherent cells were discarded and the remaining cells were cultured for 1 to 18 hr, fixed and processed for immunocytochemistry. They were incubated for 90 min in the presence of 1-3 µg/ml of the monoclonal MT1-MMP antibody 113-5BT (Fuji Chemical Industries, Ltd. Takoaka, Japan). This antibody was raised against a synthetic peptide corresponding to an amino acid sequence (CDGNFDTVAMLRGEM) (SEQ ID No.21) which differs by 1 amino acid from the corresponding rabbit sequence (V in rabbit instead of M in human at position 10). Rhodamine-labelled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) was used as secondary antibody at 200 times dilution. When incubating osteoclasts with an antibody against MT1-MMP we found fluorescence at specific points of its plasma membrane. Fluorescence did not appear when the MT1-MMP antibody was replaced by non-immune IgG. All bright signals were in the focal plane where the cells were seen in contact with their substrate. In moving cells, mainly the extremities of the lamellopodia were illuminated. In spread cells, the signals were arranged in a ring of small dots at the cell periphery. This pattern is reminiscent of podosomes. These are small extensions of the plasma membrane, that become abundant and organise in this particular way when the osteoclast is attaching. To investigate whether MT1-MMP is associated to podosomes, we stained the cell simultaneously for actin by addition of 10 mg/ml fluorescein-labelled phalloidin (Sigma, Saint Louis, MO) during the incubation with the secondary antibody.

Actin staining which is widely used to identify podosomes revealed the same ring of bright dots as shown with the anti MT1-MMP antibody. Therefore MT1-MMP appears to be localised on the podosomes. MT1-MMP staining was however somewhat more diffuse as compared to the sharp actin staining, probably because the sharp actin dots are due to bundles of actin filaments in the core of the podosome and oriented perpendicularly to the attachment surface, while MT1-MMP might be on the surface of the podosome. As expected, staining for actin illuminated also the extremities of the lamellopodia, as did the anti-MT1-MMP antibody. Similar localisations of MT-MMP were found when the osteoclast was cultured on bone slices. Thus these observations do not only demonstrate the presence on the protein level of MT1-MMP in the plasma membrane of the osteoclast, but provide new information on where exactly on the plasma membrane MT1-MMP is localised, i.e. at the level of lamellopodia and of podosomes.

Example 3

3-1 Production, purification and activation of osteoclast proteinases.

As noted in the summary of the invention, the production of osteoclast proteinases can be performed in cultures of osteoclasts or in cell lines transfected with cDNA encoding the osteoclast proteinase or a part thereof. In all cases a purification of the product is needed and in those cases where the production leads to a latent pro-form of the proteinase a subsequent activation is also needed for some purposes. Exemplifying this process, the production, purification and activation of osteoclastic pro-MMP-9 was performed according to the following descriptions:

a. Osteoclast production of pro-MMP-9

When cultured at 37°C and 5% CO_2, under serum-free conditions to avoid contamination with serum-derived proteinases and natural inhibitors of proteinases, rabbit osteoclasts secreted 92 kDa pro-MMP-9 into the culture medium. According to studies by gelatinase-zymography, addition of 40 nM of phorbol 12-myristate 13-acetate (PMA) to the cell culture increased the yield of pro-MMP-9 at least 3-fold.

b. Purification of osteoclastic pro-MMP-9

The osteoclast conditioned medium was concentrated by 10 kDa cut-off filtration (Amicon) and subsequently diluted in 2.5 mM sodium phosphate containing 0.04% Triton X-100 before application to an affinity column comprising hydroxyapatite (Bio-Rad, Hercules, CA). By this novel method for purification of MMPs, pro-gelatinases including pro-MMP-9 and pro-MMP-2 were observed to bind efficiently to the hydroxyapatite column. However, pro-MMP-9 was eluted from the column already by increasing the phosphate concentration to 5-10 mM, whereas higher concentrations (above 20 mM) of phosphate were needed to elute other pro-gelatinases and gelatinases from the column.

c. Activation of osteoclastic pro-MMP-9

The purified latent pro-MMP-9 was activated either by a traditional method based on incubation with 1 mM (4-aminophenyl)mercuric acetate (APMA) for 2-8 hrs at 37°C or by a method based on the activation of gelatinases as it is observed during analytical zymography. In the latter method the purified pro-MMP-9 was run into a slab gel by preparative SDS-PAGE. The SDS was substituted by Triton X-100 during subsequent incubation of the gel for 16 hrs in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 1% Triton X-100. A part of the gel corresponding to an electrophoretic migration distance of compounds with an approximate molecular weight of 92±5 kDa (but including the by now activated approximate 68 kDa form of MMP-9) was

excised. The active MMP-9 was electrophoretically eluted from the excised gel.

3-2 Expression and characterisation of MT1-MMP fusion proteins

The MT1-MMP cDNA fragment encoding amino acid residues Gln^{40}-Glu^{531}, Ec1 (containing the propeptide, catalytic, hinge and hemopexin, but not the signal peptide, transmembrane and cytoplasmic domains of rabbit osteoclast MT1-MMP, see Fig. 3), was PCR amplified using a 5'primer with an extra SnaBI site and a 3'primer with an extra NotI site. This fragment was inserted between the SmaI and NotI sites of the pGEX-6P-2 vector (Pharmacia). The MT1-MMP cDNA fragments encoding amino acid residues Gln^{40}-Asn^{322}, Ec2 (containing the propeptide, catalytic, and hinge, but not the signal peptide, hemopexin, transmembrane and cytoplasmic domains of rabbit osteoclast MT1-MMP, see Fig. 3) and Gln^{40}-Leu^{282}, Ec3 (containing the propeptide and catalytic but not the signal peptide, hinge, hemopexin, transmembrane and cytoplasmic domains of rabbit osteoclast MT1-MMP, see Fig. 3) were PCR amplified using 5'primers with an extra BamHI site and 3'primers with an extra XhoI site. These fragments were inserted between the BamHI and XhoI sites of pGEX-6P-2 vector (Pharmacia). The three corresponding constructs were used to express glutathione S-transferase (GST) fusion proteins in E.coli BL21 (Pharmacia).

Four overnight cultures of E.coli BL21 transformed with the three PGEX-MT1-MMP expression vectors and the PGEX vector alone (without any insert), were diluted 1:100 in 500 ml 2X YTA medium (Pharmacia). The cultures were grown at 37°C to an OD_{600}= 1.0 before adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM to induce expression. After induction for 3.5 hours at 30°C, the cells were pelleted and resuspended in 25 ml of ice-cold 1X PBS. All subsequent steps were carried out at 4°C or on ice. E.coli cells were lysed by sonication (5 bursts of 10 seconds/burst). Cellular debris was pelleted

by centrifugation at 3000 rpm after incubation with 1% Triton X-100 for 30 minutes.

The purifications were carried out by affinity chromatography using Glutathione Sepharose 4B contained in the GST Purifications Modules, according to the manufacturer's instructions (Pharmacia). The supernatants obtained after the centrifugation of the sonicated samples were absorbed on 1 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with PBS by incubation at room temperature for 30 minutes. After washing several times with 1X PBS, the fusion proteins were eluted with 900 µl of Glutathione Elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). The eluates were stored at -20°C until use.

The three fusion proteins migrated in SDS-PAGE as proteins of approx. 85, 60 and 55 kDa corresponding to their cDNA-deduced sizes of 87, 61 and 57 kDa, respectively. The fusion proteins were confirmed to be GST-MT1-MMP fusion proteins by Western-blotting using an anti-GST antibody reacting with all three proteins and an antibody to the hemopexin domain of MT1-MMP reacting with the large but not the two smaller proteins. Finally, amino acid sequencing of their propeptide domains further demonstrated that these proteins were truncated forms of MT1-MMP.

3-3 Proteolytic activity of GST-MT1-MMP fusion proteins after activation by trypsin or plasmin

In order to obtain truncated MT1-MMP in active form, Ec1, Ec2 and Ec3 were incubated with trypsin or plasmin leading to removal of the GST-part and the propeptide domain of the fusion proteins.

a. Trypsin activation

Eighty µl (20 µg approximately) of the eluted Ec1, Ec2, Ec3 and the GST tag alone were incubated at 25°C with 5 µg/ml trypsin (Promega) for 15-60 min in a final volume of 100 µl. The reactions were stopped by the addition of 50 µg/ml SBTI.

b. Plasmin activation

Twenty-five µl (7 µg approximately) of the eluted Ec1, Ec2, Ec3 and the GST tag alone, were incubated with 2.7 pmol of human plasmin (Boehringer) at 25°C for 30 minutes in a final volume of 45 µl. The reactions were stopped by the addition of 10 µM aprotinin.

c. Enzymatic assay

The proteolytic activities were evaluated by fluorescence measurements (excitation wavelength: 320 nm, emission wavelength: 387 nm) of the hydrolysis of the quenched fluorescent peptide substrate Mca-PLGL-Dpa-AR-NH_2 (Bachem) (SEQ ID No.14) after incubation at 37°C for 180 minutes in 150 mM NaCl, 10 mM $CaCl_2$, 0.05 % (v/v) Brij-35 in 50 mM Tris-HCl, pH 7.5 (see Table 1).

d. Effect of inhibitors of MMPs

Samples treated either with trypsin or plasmin in the conditions described above were preincubated for 30 minutes at 37°C in the absence or presence of the endogenous MMP inhibitors TIMP-1 (16.7 µg/ml) or TIMP-2 (16.7 µg/ml) or the synthetic MMP-inhibitor BB-94 (0.8×10^{-5} M, British Biotech). The hydrolysis of the fluorescent substrate was evaluated afterwards as described above (See Table 1).

Table 1. Hydrolysis in relative fluorescence units (RFUs) per 180 min of a synthetic substrate in the presence or absence of MMP-inhibitors by truncated forms of recombinant osteoclast MT1-MMP activated by trypsin or plasmin.

RFU/ 180 min	Trypsin activated				Plasmin activated	
	- Inhibitor	+ TIMP-1	+ TIMP-2	+ BB94	- Inhibitor	+ BB94
Ec1	139.4	ND	ND	6.7	27.5	2.9
Ec2	172.4	148	7.1	6.0	104	3.2
Ec3	9.6	ND	ND	6.9	4.1	3.8
pGEX	8.6	ND	ND	6.8	3.8	3.5

ND: not done

3-4 The cloning, recombinant expression, activation and characterisation of rabbit osteoclast MMP-12.

Due to the expression and use in cell invasion of MMP-12 in macrophages as well as the common hematopoieitic stem cell origin of osteoclasts and macrophages, we investigated whether MMP-12 was also expressed in osteoclasts. As indicated in Example 1b and shown in the present example, this was indeed the case, and we therefore expect that MMP-12 plays a similar role in osteoclast invasion and migration as it does in macrophages.

The isolation and sequencing of MMP-12 cDNA from the rabbit osteoclast cDNA library, and the subsequent steps of expression, characterisation and recombinant production of the MMP-12 fusion protein was done essentially as described for MT1-MMP cDNA (see Examples 1, 3-2 and 3-3). Briefly, the osteoclast preparations were obtained from rabbit long bones and the reverse transcribed mRNA from these osteoclasts was amplified by PCR using degenerate primers based on regions conserved in the MMP family (see Example 1b). Among several PCR fragments of the predicted sizes, one (B4) presented

homology with a sequence of human MMP-12. When a random-primed 32P-labelled probe based on the PCR product of clone B4 was used to screen a cDNA library of rabbit osteoclasts several positive clones were identified. One of these contained a cDNA insert of 1,792 bp including an open reading frame encoding a polypeptide of 464 amino acids sharing 74 %, 66 % and 65 % identity to human, rat and mouse MMP-12, respectively (see Figures 4 and 5). Based on this and further comparisons to other available protein sequences, we concluded that the isolated novel cDNA encoded the rabbit homologue of MMP-12 or of a closely related but previously unreported human MMP. The nucleotide sequence analysis of the B4 PCR fragment and rabbit MMP-12 cDNA clones from the cDNA library was done as described for MT1-MMP (see Example 1d). Using this cDNA as a probe for northern blotting, we compared the levels of expression of MMP-12 in various cells and tissues from rabbits, including calvaria, brain, placenta, lung, liver, spleen, kidney, bone stromal cells, alveolar macrophages, and purified osteoclasts. Interestingly, the level of expression in purified osteoclasts was as high as in macrophages, while expression was almost not detectable in the other cells and tissues. To investigate whether MMP-12 is also expressed in osteoclasts in vivo, we performed in situ hybridisations on sections of metacarpals of new-born rabbits, and clearly identified MMP-12 in typical osteoclasts.

For expression and characterisation of a MMP-12 fusion protein, rabbit MMP-12 cDNA containing the open reading frame (bp 58-1437, see Figure 4) was amplified by PCR using primers sense 5'-CGGGATCCCTGTGGGTCACTTCTTCT-3' (SEQ ID No.22) and antisense 5'-CCGCTCGAGCTGGCACCATTACTAGC-3' (SEQ ID No.23). The cDNA fragment was inserted into the BamHI and XhoI sites of the pGEX-6P-2 vector as described for MT1-MMP. The cDNA was shown by direct sequence analysis to lie just 5' to the GST-encoding moiety of the vector and in proper reading frame with the plasmid translation initiation site (Figure 6).

E.coli strain BL-21, transformed with pGEX-6P-2 alone (control vector) and pGEX-6P-2/MMP-12, were plated on Luria Broth (LB) agar plates with 50 µg/ml ampicillin at 37°C overnight. Single colonies were grown overnight in 50 ml of LB containing 50 µg/ml ampicillin in a shaking incubator at 30°C. Subsequently, the overnight cultures were diluted 1:100 in 400 ml of LB containing 50 µg/ml ampicillin and grown at 30°C to an OD_{600} =0.6-1.0. IPTG (Sigma) was added to a final concentration of 0.1 mM to induce production of fusion protein, and cells were maintained in culture for an additional 3 h.

Cell pellets were resuspended in 20 ml of a Tris-HCl buffer (2 mM $CaCl_2$ in 25 mM Tris-HCl, pH 7.6) containing 2 mg/ml of lysozyme and then lysed by sonication for 1 min in ice (6 bursts of 8 sec/burst). After sonication, 1 ml of 20% Triton X-100 was added and extraction continued for 30 minutes at 4°C. After centrifugation for 10 min at 20,000 x g, the fusion protein according to SDS-PAGE was localised in the pellet (estimated molecular weight approx. 75 kDa corresponding well to the cDNA-deduced size of 83 kDa).

The pellet was solubilized in 20 ml of buffer containing 8 M urea and then stirred for 1 h at 4°C. The sample was clarified by centrifugation at 40,000 x g for 30 minutes at 4°C. Subsequently, the urea was removed completely by stepwise dialysis of the supernatant against the Tris-HCl buffer. The supernatant was subjected to SDS-PAGE and proteins stained by Coomassie Brilliant Blue R250. Fusion protein expression was confirmed by Western blot using an antibody against the GST moiety. The presence of recombinant rabbit MMP-12 protein was ensured by fragmentation and subsequent amino acid sequence analysis. The elastolytic activity of the truncated recombinant MMP-12 was confirmed by elastin and gelatine zymography.

Example 4

Assessment of the role of osteoclast MMPs in osteoclast migration.

In bone tissue cultures, we previously showed that MMPs are very important for the recruitment of osteoclasts to future resorption sites (Blavier and Delaissé, 1995), but until now osteoclast purification techniques did not allow the demonstration of whether these MMPs were from osteoclasts or other cells. We therefore developed an experimental model in order to address the latter question. Briefly, we seeded purified or non-purified osteoclasts on membranes (12 µm pore size) coated with type I collagen, and followed their migration to the lower surface of the membranes after an overnight culture in the absence or presence of MMP inhibitors. We found that not only when using non-purified osteoclast preparations, but also when using purified preparations, osteoclasts could extend cell processes into the pores of the membranes and spread over the lower surface of the membranes. This migration process was inhibited by MMP inhibitors of both the synthetic pseudo-substrate type (RP59794 and BB94) and the natural type (TIMP-2) (Figure 7). This indicates that osteoclasts themselves can overcome a collagen barrier by migrating through it via an MMP dependent pathway, without the participation of other cells.

In order to evaluate how important MMPs are for this migration as compared to other proteinases, we also tested inhibitors of other classes of proteinases on this migration. Cysteine proteinase inhibitors that are potent inhibitors of the degradation of bone matrix in the subosteoclastic resorption zone, affected only slightly the migrations, whereas a serine proteinase inhibitor was without any effect (Figure 7). Thus MMPs play a unique role in osteoclast migration as compared to other proteinases.

In order to confirm the role of MMPs in an overall migration/resorption sequence, we seeded purified osteoclasts on dentine slices that were coated or not with type I collagen, cultured them overnight in the presence and absence of MMP inhibitor and followed the formation of pits in the dentine slices. We found that the MMP inhibitor inhibited pit formation only in the collagen coated dentine slices (Figure 8). This indicates clearly that the role of MMPs is on the migration of the osteoclasts to their future resorption site, and not on resorption itself.

Example 5

Preparation, characterisation and application of antibodies to MMPs

Two approaches were used for the production of anti-MMP antibodies. In one approach, intact or truncated, native or recombinant MMP was used as an immunogen (see a, below) and in the other approach synthetic peptide mimicking a specific MMP-region was used as an immunogen after having been conjugated to a larger carrier protein (see b-d, below):

a. Preparation and use of intact or truncated MMP immunogens

As an example of the first approach, pro-MMP-9 purified from osteoclast cultures as described in Example 3-1 was used for immunisation either in its latent form or after activation by APMA or by in-gel treatment with SDS/Triton X-100. The preparations of pro-MMP-9 and MMP-9 were injected intra-peritoneally every third week in female BALB/c-CF1 murine hybrids. A final booster immunisation of the protein without adjuvant was given 3 days prior to splenectomy. The spleen cells were fused with P3-X-63-Ag8.653 myeloma cells in the presence of 50% polyethylene glycol 4000 and the resulting hybridoma cells propagated and cloned according to

standard procedures. Monoclonal antibody was purified from the conditioned medium of hybridoma cultures by using protein A affinity chromatography.

b. Preparation of MMP-mimicking conjugated peptide immunogens

Based on the amino acid sequence of osteoclastic MT1-MMP (Figures 1 and 2) and sequences available for other members of the MMP family, such as MMP-9 and MMP-12, femtameric sequences (i.e. polypeptide sequences of 15 amino acids) were selected due to:

1. their specificity for one member of the MMP family when compared to other members;
2. their putative properties as immunogens according to computer-based algorithms used for analyses of their hydrophilicity, their position and their expected secondary structure in the intact MMP; and
3. their conservation i.e. their possible sequence identity or similarity in corresponding regions of the same MMP in the human, rabbit and mouse species.

Corresponding to the selected femtameric sequences, femta-peptides were synthesised by using Fmoc-amino-acids-O-pentafluorophenyl-esters in the presence of catalytic amounts of 3,4-dihydro-4-oxo,1,2,3-benzotriazin-3-yl in a fully automated custom made peptide synthesiser.

The femta-peptides were coupled to a proteinaceous carrier molecular (thyroglobulin). Briefly, thyroglobulin and glutaric anhydride (1:2 w/w) were incubated for 2 hrs at 20°C in 0.1 M sodium borate, pH 9.0 and subsequently desalted on a Nap 10/Sephadex G-25 column (Pharmacia) and dried by vacuum centrifugation. The carrier was resolubilized in 0.01 M sodium phosphate, pH 5.0 and incubated for 3 min at 20°C with equal volumes of 5 mg/ml freshly prepared 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (CDI). The CDI-activated thyroglobulin was incubated 4 hrs at 20°C in equal volumes and amounts (w/w) with the femta-peptide in 0.2 M

sodium phosphate, pH 9.0. The thyroglobulin/CDI/femta-peptide conjugates were dialysed and their protein content determined.

c. *Production of polyclonal antibodies by use of conjugated peptide immunogens*

The thyroglobulin/CDI/femta-peptide conjugates were mixed with Freunds incomplete adjutant and injected intramuscularly once per month in female New Zealand White rabbits. Blood was collected and the immunoglobulin fraction purified from the corresponding serum by ammonium sulphate precipitation.

d. *Production of monoclonal antibodies by use of conjugated peptide immunogens*

The thyroglobulin/CDI/femta-peptide conjugates were mixed with Freunds incomplete adjuvant and injected intraperitoneally every third week in female BALB/c-CF1 murine hybrids. A final booster immunisation of the conjugate without adjuvant was given 3 days prior to splenectomy. The spleen cells were fused with P3-X-63-Ag8.653 myeloma cells in the presence of 50% polyethylene glycol 4000 and the resulting hybridoma cells propagated and cloned according to standard procedures. Monoclonal antibody was purified from the conditioned medium of hybridoma cultures by using protein A affinity chromatography.

e. *Characterisation and application of specific anti-MMP antibodies*

The antisera and monoclonal antibodies were selected and initially characterised by enzyme-linked immunosorbent assay (ELISA) based on 96-well polystyrene plates coated with either purified intact or truncated MMPs or homologous or heterologous conjugated femta-peptides. As indicated above, antisera and monoclonal antibodies showing MMP-

specificity according to the initial characterisation by ELISA have several applications. One example is their use in immunohistochemical identification of MMP-expression on the protein level by incubation of an anti-MMP antibody with bone cells or tissues. As described in Example 2d, the binding of a monoclonal antibody raised by immunisation with a MT1-MMP mimicking peptide to the actin-rich membranous areas of an osteoclast shows that MMP-antibodies not only are tools of central importance to the identification of the cells which produce a particular MMP, but also can demonstrate the cellular localisation of a MMP and thereby aid in the clarification of its biological role.

Sera from mice immunised with the thyroglobulin-conjugated femta-peptide RSGAPVDQMFPGVPL (SEQ ID No.13) corresponding to a region in the hemopexin domain of rabbit MMP-9 and either boosted with the same conjugated peptide or with purified native osteoclast proMMP-9 showed inhibitory effects to activated MMP-9. The analysis was done by a fluorometric enzymatic assay based on pre-incubation of diluted sera with MMP-9 for 30 min at 37° before incubation with the synthetic peptide-like substrate Mca-PLGL-Dpa-AR-NH_2 (Bachem) (SEQ ID No.14) for 30 minutes at 37° in 150 mM NaCl, 10 mM $CaCl_2$, 0.05 % (v/v) Brij-35 in 50 mM Tris-HCl, pH 7.5 (see Figure 9).

Example 6

Production of non-immunoglobulin inhibitors of osteoclast proteinases.

Production of non-immunoglobulin inhibitors of osteoclast proteinase aimed at two main type of agents, one being peptide or peptide-mimicking proteinase inhibitors another being antisense probes specifically binding to osteoclast proteinase mRNA. The peptide and peptide mimicking agents were produced by two methods: a technology based on PEGA bead peptide substrate and inhibitor libraries

(see a-c, below), the other being based on positional combinatorial peptide inhibitor libraries (see d-e, below). The design and use of antisense probes is described in f (see below):

a. Identification of MMP substrates by PEGA bead libraries

According to previous descriptions (Meldal *et al*, 1994[10]), two PEGA bead peptide substrate libraries were generated consisting each of approx. 10^6 different beads. Each bead contained many copies of a single sequence: ^{N}X1-X2-Y(NO_2)-X3-X4-X5-X6-X7-X8-K(Abz)^{C}-PEGA (PEGA bead substrate library A) or ^{N}X1-X2-Y(NO_2)-X3-X4-X5-X6-K(Abz)^{C}-PEGA (PEGA bead substrate library B), where X1 to X8 are amino acids varying randomly from bead to bead, and Y(NO_2) and K(Abz) is a quenching 3-nitrotyrosine and a fluorogenic lysine(2-aminobenzoic acid), respectively. The libraries were incubated at 37°C with purified and activated osteoclast proMMP-9 (approx. 0.1 μM) and fluorogenic beads subsequently isolated by a micropipette under fluorescence microscopy. The isolated beads were analysed by an amino acid sequencer.

The incubation of the randomised PEGA-bead substrate libraries lead to identification of 15 clearly fluorescent beads, indicating a specific cleavage of their corresponding peptide in contrast to the millions of other structures in the libraries. The amino acid sequences of the cleaved substrates showed some consistency (see Table 2). In particular a proline at the third position (P3) towards the N-terminal from the cleavage site was highly conserved.

Table 2. Amino acid sequences and cleavage site of quenched fluorogenic peptide substrates identified on PEGA bead libraries (A) and (B).

	Cleavage site											
Bead #	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
A2		S	K	Y'	P	J	A	L	F	F	K'	
A3	S	R	Y'	?	P	J	G	L?	T	K'		
A5	W	G	Y'	E	A	J	G	F	T	K'		
B1		A	R	Y'	P	K	K	V	K'			
B2		N	J	Y'	P	J	J	Y	K'			
B3		Y	I	Y'	P	J	M	L	K'			
B5		R	P	Y'	P	Y	?	K	K'			
B6		L	K	Y'	P	K	?	L	K'			
B7				F	A	Y'	J	M	R	?	K'	
B8				P	A	Y'	M	K	K	M	K'	
B9					P	L	Y'	M	S	?	J	K'
B10					P	V	Y'	M	R	G	J	K'
B11					V	R	Y'	L	H	G	J	K'

b. *Synthesis and characterisation of soluble peptides analogous to peptides identified by the PEGA bead substrate library technology*

To further evaluate the results observed for peptide substrates bound to PEGA beads, a series of soluble peptide substrates was synthesised by multiple column peptide synthesis (Meldal *et al*, 1994[11]). The amino acid sequences of these putative soluble substrates were based on either single peptide substrate sequences or consensus sequences from the PEGA bead studies. The hydrolysis by MMP-9 and other MMPs of the soluble peptides was analysed by a standard fluorometric assay (excitation: 320 nm, emission:

425 nm).

As an example, one of the fluorescent beads (A2 in Table 2) isolated from PEGA bead peptide substrate library (A) contained two similar peptides with the sequences S-K-Y(NO_2)-P-J-A-L-F-F-K(Abz)-PEGA (SEQ ID No.2) and L-F-F-K(Abz)-PEGA (SEQ ID No.24) indicating hydrolysis by osteoclastic MMP-9 of the novel peptide-mimicking substrate S-K-Y(NO_2)-P-J-A-L-F-F-K(Abz) (SEQ ID No.2) at the P1-P1' position: A-L. Based on this information several soluble quenched fluorogenic peptides were synthesised (e.g., CL-1 and CL-6, see Table 3 and Figures 10 and 11). By a similar strategy for the other amino acid sequences of substrates identified in the PEGA bead substrate libraries A and B, the first 30 soluble quenched fluorogenic peptide substrate candidates for MMP-9 (named CL-1 to CL-30) were synthesised by multiple column peptide synthesis. Their individual kinetic properties (k_{cat} and K_m) were determined by incubation at 37°C with MMP-9 and recombinant truncated MT1-MMP of osteoclast origin, and as controls recombinant truncated MMP-1 and -3; the osteoclast cysteine proteinase, cathepsin K; and the broad-reacting proteinase, subtilisin. Several of the hitherto produced 30 synthetic substrates showed a high selectivity for one or more MMPs; no or very low reactivity with cathepsin K; and k_{cat}/K_m ratios up to 50-fold higher for MMP-9 than for subtilisin. This was particularly clear for the peptide substrates CL-21, CL-25 and CL-29 (see Table 4). Further peptide substrate designing based on the sequence information obtained from both those of the 30 peptides which were cleaved specifically by MMP-9 and those that were not, can be expected to lead to other even more selective synthetic MMP substrates.

For some of the 30 soluble putative peptide substrates, the kinetic behaviour was different from what was expected according to the hydrolysis of the corresponding peptide immobilised on the PEGA bead. E.g., the putative substrate, CL-1, was inhibitory to MMP-9 as would have been expected for a pseudo-substrate, i.e. with a low K_m(3.4 µM) and a low k_{cat}/K_m (250 $M^{-1}s^{-1}$) (see Table 3).

Table 3. Kinetic parameters for the hydrolysis of three established soluble MMP-9 substrates (B, MR1, MR2) and two soluble substrates (CL1, CL6) designed according to results from PEGA bead library (B).

Name	Sequence (and MMP-9 cleavage site: *)		K_M (μM)		K_{cat}/K_M ($M^{-1}s^{-1}$)	
			MMP-9	Subtilisin	MMP-9[a]	Subtilisin
B	Mca-P-L-G*L-Dpa-A-R-NH_2	(1)	7.4	21.3	$9.1*10^5$	$1.1*10^7$
MR1[b]	Abz-G-P-L-G*L-Y(NO_2)-A-R-NH_2	(2)	7.7	1.6	$3.1*10^5$	$2.1*10^6$
MR2[b]	Abz-G-P-L-G*L-L^{ncr}-A-R-Y(NO_2)NH_2	(3)	7.3	4.8	$9.0*10^4$	$9.6*10^6$
CL1[c]	Abz-S-K-Y-P-J-A*L-F-Y(NO_2)-D	(4)	3.4	7.5	$2.5*10^2$	$1.6*10^3$
CL6[c]	S-K-Y(NO_2)-P-J-A*L-F-F-K(Abz)-D	(5)	20.0	9.5	$3.1*10^2$	$6.4*10^4$

a Due to the lack of a proper MMP-9 standard, the estimation of k_{cat} for MMP-9 was not exact.
b Analogues of peptide B (Bachem M-1895)
c Based on the isolated fluorogenic bead: A2 (see Table 2).

1. SEQ ID No.14
2. SEQ ID No.25
3. SEQ ID No.15
4. SEQ ID No.16
5. SEQ ID No.2

Table 4. Kinetic parameters for the hydrolysis of three soluble selective MMP-9 substrates (CL-21, CL-25 and CL-29) designed according to results from PEGA bead substrate library (B). The kinetic parameters are k_{cat}/K_m in $\mu M^{-1} \times min^{-1}$ and relatively (%) to the corresponding value for MMP-9.

Peptide	Sequence	Bead #[a]	MMP-9	Subtilisin	Cathepsin K	MMP-1	MMP-3	MT1-MMP[b]
CL-21	Y'PLJMKGK'G	B8/B9	5.5 100%	0.09 2%	0 0%	0.28 5%	0.05 1%	0 0%
CL-25	NJY'PJJYK'G	B2	0.08 100%	0 <6%	0 <6%	0 <6%	0 <6%	0 <6%
CL-29	Y'PJJMK'GJG	B2/B10	0.38 100%	0.01 3%	0 <2%	0 <2%	0.01 3%	0.01 3%

a The synthetic peptides were designed according to amino acid sequences of peptides from those beads of the PEGA-bead substrate libraries that became fluorescent upon incubation with MMP-9 (Bead #, see Table 2).

b Represented by the trypsin-activated form of the truncated recombinant MT1-MMP, Ec2.

CL-21 = SEQ ID No.26
CL-25 = SEQ ID No.27
CL-26 = SEQ ID No.28

c. *Identification of MMP inhibitors by PEGA bead libraries*

According to previous descriptions (Meldal *et al*, 1994[11], Meldal & Svendsen, 1995[12], Meldal *et al*, 1997), a PEGA bead peptide inhibitor library (I) was generated consisting of approx. 10^6 different beads, each containing many copies of a single well-defined substrate sequence as well as many copies of a randomly generated putative inhibitor sequence: ^{N}X1-X2-X3-D-X4-X5-X6-V^{C}-PEGA, where X1 to X6 are L-amino acids varying randomly from bead to bead, and D is a D-amino acid varying randomly from bead to bead. The library was incubated at 37°C with active MMP-9 and beads remaining quenched (i.e. dark compared to the majority of brightly fluorescent beads) were isolated by a micropipette under fluorescence microscopy. The isolated beads were analysed by an amino acid sequencer and since the substrate sequence was not degraded by the Edman degradation due to prior acylation at the N-terminus, the sequences obtained corresponded to potential peptide-mimicking MMP-9 inhibitors.

A novel type of PEGA bead inhibitor library was developed in order to identify peptide substrate mimicking MMP-inhibitors with a phosphinate instead of a peptide bond at the susceptible cleavage site (i.e. between the expected P1 and P1' sites of the corresponding substrate). Two PEGA bead phosphinate inhibitor libraries (IIa and IIb) were generated. Each library consisted of approx. 10^6 different beads, and each PEGA bead contained many copies of a single well-defined substrate sequence as well as many copies of a randomly generated putative inhibitor sequence: ^{N}X1-X2-J^{P}/^{C}M-X3-X4-"Linker"-EGA (in IIa) or ^{N}X1-X2-G^{P}/^{C}L-X3-X4-"Linker"-PEGA (in IIb), where X1 to X4 are L-amino acids varying randomly from bead to bead, and J^{P}/^{C}M and G^{P}/^{C}L is the phosphinate pseudo dipeptide used in library IIa and IIb, respectively (see Figures 12-15). The design of the first two phosphinate pseudo dipeptides was based on the identity of suitable P1 and P1' amino acids in newly developed and existing MMP-9 substrates. Other combinations of pseudo amino acids around the phosphinate bond will also be

investigated according to the findings of MMP selective peptide substrates by use of e.g. PEGA bead substrate libraries.

d. Positional combinatorial peptide inhibitor libraries

As an alternative to using the PEGA bead peptide libraries for identification of potential MMP inhibitors, 20 different positional combinatorial peptide inhibitor libraries (Houghten et al, 1991[7]) were produced using pentapeptides constructs X-X-D-X-X, where D is the D-form of one of the 20 common amino acids (except glycine) or hydroxyproline, and X is a randomly varying natural L-form of one of the 20 common amino acids or hydroxyproline. The peptide libraries were purified by high performance liquid chromatography in order to remove salts and other substances which were toxic to bone tissue cultures before being tested for inhibitory effects on osteoclast migration and bone resorption in murine foetal metatarsal cultures. Each of the 20 libraries contained 30 μmol pentapeptides composed of up to 21^4(194,481) different structures.

e. Murine foetal metatarsal cultures for studying osteoclast migration and resorption in vitro

$^{45}Ca^{2+}$ pre-labelled metatarsals isolated from 17 day old NRMI mouse foetuses were used as an organ culture model (Blavier & Delaissé, 1995[3]). Briefly, foetal bones were labelled by subcutaneous injection of $^{45}Ca^{2+}$ into pregnant mice at day 16 of gestation. Foetal metatarsals isolated on the following day thereby comprised ^{45}Ca-labelled calcified matrix developed *in uteri* between day 16 and 17. In the periosteum surrounding the calcified matrix numerous osteoclast precursors cells were present. Corresponding to the development of bone and bone marrow in metatarsals *in vivo*, subsequent cultivation of the isolated metatarsals in BGJb medium containing 30 nM 1a,25 dihydroxy-vitamin D3 and 0.1% Albumax for 1 to 7 days resulted in differentiation, fusion and migration of the osteoclast precursor cells leading to the presence of mature osteoclasts in the central calcified matrix where the resorbed bone and formed the primitive marrow cavity. The development

and bone resorbing activity of the osteoclasts was estimated by measurement of the release of $^{45}Ca^{2+}$ into the culture medium at various time points and by microscopic inspection of the positioning in the cultured metatarsals of osteoclasts stained for tartrate-resistant acid phosphatase. The general MMP inhibitor, RP59794 which has been shown previously to inhibit the migration of osteoclasts and thereby reduce the release of $^{45}Ca^{2+}$ in the metatarsal culture model (Blavier & Delaissé, 1995[3]) was included as a positive control in all experiments.

The effect of the 20 X-X-D-X-X combinatorial libraries on bone resorption was evaluated by measuring the change (%) in accumulated ^{45}Ca-release into the conditioned medium of the treated metatarsal culture relatively to the ^{45}Ca-release of the corresponding non-treated metatarsal culture originating from the other leg of the same foetus at Day 1, 2 and 4. Each library was tested in 4 independent metatarsal cultures in the same experiment and in some cases the experiment was repeated.

Each of the 20 libraries was used in a concentration of 3 mM total peptide corresponding to a concentration of approx. 15 nM for each of the 194,481 structures in a library. The majority of the 20 libraries did not significantly affect the bone resorption, whereas 1 of the 20 libraries (D=ile) showed significant reductions in the ^{45}Ca-release at Day 4 (see Figure 16), and most importantly 2 of the 20 libraries (D=leu and D=trp) showed significant inhibitions at both Day 2 and Day 4 (see Figure 16 and Table 5).

Table 5 Change (in %) of ^{45}Ca-release due to the addition of a X-X-D-X-X combinatorial library to 4-day metatarsal cultures

Library	Day 0-1	Day 0-2	Day 0-4
X-X-trp-X-X	0% (ns)	-20% (0.02)	-40% (0.05)
X-X-leu-X-X	0% (ns)	-34% (0.0001)	-48% (0.0005)

The p-values express the level of significance of the changes between the treated and corresponding non-treated group (n=4 for each).

Further investigations of the X-X-trp-X-X library was done by performing a second screening of 28 libraries with a selected variation at one of the 4 X-positions. The following conformations were used U-X-trp-X-X, X-U-trp-X-X, X-X-trp-U-X and X-X-trp-X-U, where U is a random mixture of L-amino acids belonging to a specific undergroup: U1: K and R (n=2); U2: H, Y, F and W (n=4); U3: E and Q (n=2); U4: T, D, S and N (n=4); U5: C, V, L, I and M (n=5); U6: P and J (n=2); and U7: A, G (n=2). Each of the 28 libraries was used in a concentration of 1.6-4.0 mM total peptide, corresponding to approx. 85 nM for each of the 18,522 to 46,305 structures in a library. The majority of the 28 libraries did not significantly affect the bone resorption, whereas 5 of the 28 libraries showed significant and/or marginally significant reductions in the ^{45}Ca-release at Day 1, 2 and/or 4 (see Table 6).

Table 6. Change (in %) of ^{45}Ca-release due to the addition of a U-X-trp-X-X, X-U-trp-X-X, X-X-trp-U-X or X-X-trp-X-U combinatorial library to 4-day metatarsal cultures

Library	Day 0-1	Day 0-2	Day 0-4
U5-X-w-X-X	-20% (0.28)	-21% (0.12)	-15% (0.11)
X-U2-w-X-X	-15% (0.35)	-23% (0.05)	-18% (0.15)
X-U5-w-X-X	-43% (0.007)	-28% (0.04)	-11% (0.05)
X-X-w-U2-X	-23% (0.21)	-20% (0.003)	-16% (0.001)
X-X-w-X-U5	-23% (0.15)	-24% (0.0008)	-15% (0.07)

Further investigations of the U5-X-trp-X-X, X-U2/5-trp-X-X, X-X-trp-U2-X and X-X-trp-X-U5 libraries was done by performing a third screening of 23 libraries with a single variation at one of the 4 X-positions. The following conformations were used Z5-X-trp-X-X, X-Z2/5-trp-X-X, X-X-trp-Z2-X and X-X-trp-X-Z5, where Z2, Z5 or Z2/5 is a single L-amino acid belonging to undergroup(s) U2, U5 or U2 and U5, respectively. With a few exceptions, each of the 23 libraries was used in concentration of 3.2 mM total peptide, corresponding to approx. 340 nM for each of the 9,261

structures in a library. More than half of the 23 libraries did not significantly affect the bone resorption, whereas 11 of the 23 libraries showed significant and/or marginally significant reductions in the ^{45}Ca-release at Day 1, 2 and/or 4 (see Table 7).

Table 7 Change (in %) of ^{45}Ca-release due to the addition of a Z5-X-trp-X-X, X-Z2/5-trp-X-X, X-X-trp-Z2-X or X-X-trp-X-Z5 combinatorial library to 4-day metatarsal cultures

Library (Conc)	Day 0-1	Day 0-2	Day 0-4
C-X-trp-X-X (3.2 mM)	-22% (0.09)	-38% (0.03)	-34% (0.006)
V-X-trp-X-X (3.2 mM)	- 6% (0.33)	-30% (0.17)	-23% (0.11)
L-X-trp-X-X (3.2 mM)	-23% (0.07)	-32% (0.01)	-20% (0.06)
X-W-trp-X-X (3.2 mM)	-19% (0.22)	-26% (0.003)	-22% (0.08)
X-Y-trp-X-X (3.2 mM)	-26% (0.04)	-27% (0.06)	-18% (0.17)
X-F-trp-X-X (3.2 mM)	-20% (0.19)	-33% (0.06)	-23% (0.09)
X-C-trp-X-X (3.2 mM)	-39% (0.02)	-18% (0.14)	-10% (0.13)
X-L-trp-X-X (3.2 mM)	-19% (0.39)	-26% (0.07)	-24% (0.20)
X-X-trp-Y-X (3.2 mM) (0.8 mM) (0.8 mM)	-25% (0.05) -45% (0.07) -39% (0.003)	-48% (0.0003) -26% (0.14) -30% (0.02)	-38% (0.0006) - 8% (0.22) -18% (0.03)
X-X-trp-X-L (3.2 mM)	-12% (0.34)	-34% (0.21)	-17% (0.18)
X-X-trp-X-M (3.2 mM)	-26% (0.21)	-26% (0.14)	-18% (0.03)

In an early attempt to identify single peptide inhibitory structures a fourth screening was performed on 20 peptides of the structure C/V/L-Y/F/W/C/L-trp-Y-M/L considered to be likely candidates according to the results in the 3rd screening. Each of the 20 single structure peptides was used in a concentration of 13 µM. The majority of the 20 peptides did not significantly affect the bone resorption, whereas 5 of the 20 structures showed significant and/or marginally significant reductions in the ^{45}Ca-release at Day 1, 2 and/or 4 (see Table 8). Even better single peptide inhibitors will be obtained upon further investigations based on the data from the first 4 screenings. Particularly further investigations of X-X-trp-Y-X

combinatorial libraries and a similar screening programme for X-X-leu-X-X seem promising.

Table 8. Change (in %) of ^{45}Ca-release due to the addition of a single peptide structure with the sequence C/V/L-Y/F/W/C/L-trp-Y-M/L to 4-day metatarsal cultures

Structure	Day 0-1	Day 0-2	Day 0-4
C-L-w-Y-L	-30% (0.02)	-22% (0.03)	-15% (0.005)
C-L-w-Y-M	-29% (0.06)	-26% (0.05)	-13% (0.40)
C-Y-w-Y-L	-17% (0.008)	-18% (0.009)	-12% (0.11)
V-Y-w-Y-M	-17% (0.21)	-21% (0.04)	-15% (0.02)
L-F-w-Y-L	-34% (0.003)	-37% (0.007)	-26% (0.04)

f. Design and use of antisense probes to MMPs.

Antisense oligonucleotide probes against various MMPs were produced in order to study their influence on bone metabolism and osteoclast biology in bone cell and tissue cultures as well as in animal models. The antisense oligonucleotide probes were designed by choosing sequences which were specific to a particular MMP and showing as little as possible similarity to any predictably relevant mammalian genes. In all cases a sense probe and/or a so-called scrambled probe was used as negative controls for comparison to the antisense probe. In order to stabilise the probes, some were produced in a partially phosphorthiolated form to protect them against degradation by nucleases (phosphate bonds which are phosphorthioate bonds instead of normal phosphordiesters are marked with a * in the diagram below). In order to make the delivery of the probes to the interior of osteoclasts some of the probes were included in liposomes before application to the cell or tissue cultures.

The strategy in this type of experiments is exemplified by results from design, synthesis and testing of antisense probes to mouse and rabbit MMP-9.

Two sets of probes (17-mers) to murine MMP-9 are shown in the Table below:

Table 9: Selected probes for use in experiments with MMP-9 expression in murine cells and tissues:

First set	5'-T*G*GTATGTGGTCTGT*G*T	Scrambled	(SEQ ID No.29)
	5'-T*G*TGGTTCAGTTGTG*G*T	Antisense	(SEQ ID No.30)
	5'-A*C*CACAACTGAACCA*C*A	Sense	(SEQ ID No.31)
Second set	5'-G*GAC*T*CA*TGG*TGAG*G*A*C	Antisense	(SEQ ID No.32)
	5'-C*GGA*T*ACAGG*TG*TC*G*G*A	Sense	(SEQ ID No.33)

The probes were used in the murine metatarsal system described in Example 6e and in a murine pre-osteoclast culture system. The latter was based on unfractionated bone cells isolated from 12 day old mice and cultured for 7 days in the presence of 5% fettle calf serum in order to eradicate all multinucleated osteoclast leaving only stromal cells and osteoclast precursors. Upon subsequent culture of approximately 10 days in the presence of 2 µg/ml PGE_2, new mature osteoclasts were formed. The continuous differentiation of pre-osteoclasts to mature osteoclasts in this culture system correlated well to production of pro-MMP-9 according to gelatinase zymographical studies of the corresponding conditioned medium. For both test systems, the probe was added to the culture medium in a concentration varying between 1 and 10 µg/ml and the medium was renewed every day.

Seven antisense probes (14- to 18-mers) to rabbit MMP-9 were constructed as shown in the Table below:

Table 10: Selected probes for use in experiments with MMP-9 expression in rabbit cells and tissues:

Probe 1 (start codon)	G*T*C*TGG*GGC*T*CA*TGG*T*G*A (SEQ ID No.34)
Probe 2 (start codon)	G*G*CT*CA*TGG*TGA*G*G (SEQ ID No.35)
Probe 3 (start codon)	G*G*GC*T*CA*TGG*TG*AGG*G*G*A (SEQ ID NO.36)
Probe 4 (start codon)	C*T*CA*TGG*TG*AGG*GGA*G*C*A (SEQ ID No.37)
Probe 5 (start codon)	A*T*GG*TG*AGG*GGAG*CA*G*C*G (SEQ ID No.38)
Probe 6 (stem loop)	A*G*GT*GAG*TGG*CGT*CA*C*C*G (SEQ ID No.39)
Probe 7 (stem loop)	G*C*TGT*CA*AAG*T*TGGA*A*G*T (SEQ ID No.40)
Scrambled 1	G*G*CC*T*C*TAC*CG*CAACT*G*C (SEQ ID No.41)
Scrambled 2	G*G*C*C*T*C*TAGG*GGAAC*T*G*C (SEQ ID No.42)

Five of the antisense probes spanned the start codon of the mRNA and two targeted single stranded loops (identified by mRNA secondary structure prediction algorithms) within the translated region.

Testing of the effects of the antisense and scrambled probes to rabbit MMP-9 was performed in osteoclasts isolated from long bones of 8 to 10 days old rabbits. The osteoclasts were cultured on bovine bone slices in 5 % foetal calf serum, with renewal of media and oligonucleotides every day. The results were evaluated by quantification of MMP-9 in gelatinase zymography and by studies of osteoclast morphology and numbers as well as quantification of the secretion of tartrate-resistant acid phosphatase into the conditioned medium of the osteoclast cultures by enzymatic assay.

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phenotyping by random cDNA sequencing. Bone 17:111-119. 1995.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Center for Clinical & Basic Research
(B) STREET: Ballerup Byvej 222,
(C) CITY: Ballerup
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(F) POSTAL CODE (ZIP): DK-2750

(ii) TITLE OF INVENTION: The Use of Proteinase Inhibitors for the
Prevention or Reduction of Bone Resorption

(iii) NUMBER OF SEQUENCES: 23

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9615976.9
(B) FILING DATE: 30-JUL-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/product= "x is hydroxyproline"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Lys Tyr Pro Xaa Ala Leu Phe Phe Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:3
(D) OTHER INFORMATION:/product= "X is Y(NO2)"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/product= "X is hydroxyproline"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:10
(D) OTHER INFORMATION:/product= "X is K(Abz)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Lys Xaa Pro Xaa Ala Leu Phe Phe Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2546 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryctolagus cuniculus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TAACGCAGAG TTACATATAC ATACCTGGGG GGGGGGGGGG GGTTCTACTA
ATGTAGCGTA 60

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TCTAAATGTT 120

AGATTTTTAT TTATTTCCTA TATACTTTAC TTATTATTTA TTTCTTTGAT
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TTAGCACACG CAAACTTACA ACACAGAGTT CTATCCTATC CCTATTAGTT
ACCTTATTAG 240

TTACCTATTA GTTACCTTAT TAGTTACCTA TTAGTTTTAC CTTATTAGTT
TTACCTATTA 300

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GAATCTACTA 360

AATGTTAGCC GCTAGGAATC CAAAGTCGGT GCCTCCGGAA GACAAAGGCG
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AGATGGCGGC GCGACCCCTA GGCGAGGGCC CCGCCGCGGA ACCGCCCAGC
CCGGCTGACC 480

CCGACGGTCG CGGACCATGT CTCCCGCCCC ACGACCCTCC CGCAGGCTCC
TGAACTCCCC 540

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TACGCACCCA 660

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GAGGTTCTAC 720

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AGGCGCCCCC 780

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GAAGGAAGCG 840

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CATCCAGAAT 900

TACACCCCCA AGGTGGGCGA ATATAAAATC TAAATGTTAG GCCACATTCG
AGGCCATTCG 960

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GAAGCCGACA 1080

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TGGCTTCCTG GCCCACGCCT ACTTCCCGGG CCCCAACATT GGAAAACTCT
ACTAAATGTT 1200

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GCTGGGCCAT 1320

GCCCTGGGCA ACTGGAGCAC TCCAATGACC CCTCAGCCAT CATGGCACCG
TTTTACCAAT 1380

GGATGAAGAC ACAGAGAACT TCGTGCTGCC TGATGATGAC CGCCGGGGCA
TCCAACAGCT 1440

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GACAACCAAT 1500

CCCGGACTTT TATCCCCGAT AAGCCCAGGA ACCCCACCTA CGGGCCCAAC
ATCAATGTGA 1560

CGGGAACTTT GACACTGTGG CCGTGCTCCG AGGAGAGATG TTTGTCTTCA
AAAGGAGCGC 1620

TGGTTCTGGA GGGTGAGGAA CAACCAAGTG ATGGACGGCT ACCCAAAATG
CCCATCGGCC 1680

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AGGATGGCAA 1740

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CCTGGAGCCT 1800

GGCTACCCCA AGCACATCAA GGAGCTGGGC CGAAACTCTA CTAAATGTTA
GGGGCTTCCC 1860

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ACTAAATGTT 1920

AGAACCTACT TCTTCCGGGG AAACAAGTAC TACCGATTCA ACGAGGAGCT
CAGGGCAAAG 1980

TGGACAGCGA GTACCCCAAG AACATCAAAG TGTGGGAAGG CATCCCCGAG
TCTAACCCAG 2040

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AAACAAATAC 2100

TGGAAATTCA ACAACCAGAA GCTGAAGGTG GAGCCCGGCT ACCCCAAAAG
TCCGCCCTGC 2160

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AGGAAGAGAC 2220

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CGCGGCCGCC 2280

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GCGGTCTTCT 2340

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CCCTGCTGGA 2400

CAAGGTCTGA CCCCCACCGC TGGCCAACAC CCACTCCCAC CGCAAGGACT
TTGCTCTTCC 2460

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryctolagus cuniculus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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20 25 30

Phe Ser Pro Glu Ala Trp Leu Gln Gln Tyr Gly Tyr Leu Pro Pro Gly
35 40 45

Asp Leu Arg Thr His Thr Gln Arg Ser Pro Gln Ser Leu Ser Ala Ala
50 55 60

Ile Ala Ala Met Gln Arg Phe Tyr Gly Leu Arg Val Thr Gly Lys Ala
65 70 75 80

Asp Thr Asp Thr Met Lys Ala Met Arg Arg Pro Arg Cys Gly Val Pro
85 90 95

Asp Lys Phe Gly Ala Glu Ile Lys Ala Asn Val Arg Arg Lys Arg Tyr
100 105 110

Ala Ile Gln Gly Leu Lys Trp Gln His Asn Glu Ile Thr Phe Cys Ile
115 120 125

Gln Asn Tyr Thr Pro Lys Val Gly Glu Tyr Ala Thr Phe Glu Ala Ile
130 135 140

Arg Lys Ala Phe Arg Val Trp Glu Ser Ala Thr Pro Leu Arg Phe Arg
145 150 155 160

Glu Val His Tyr Ala Tyr Ile Arg Asp Gly Arg Glu Lys Gln Ala Asp
165 170 175

Ile Met Ile Phe Phe Ala Glu Gly Phe His Gly Asp Ser Thr Pro Phe
180 185 190

Asp Gly Glu Gly Gly Phe Leu Ala His Ala Tyr Phe Pro Gly Pro Asn
195 200 205

Ile Gly Gly Asp Thr His Phe Asp Ser Ala Glu Pro Trp Thr Val Arg
210 215 220

Asn Glu Asp Leu Asn Gly Asn Asp Ile Phe Leu Val Ala Val His Glu
225 230 235 240

Leu Gly His Ala Leu Gly Leu Glu His Ser Asn Asp Pro Ser Ala Ile
245 250 255

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260 265 270

Asp Asp Asp Arg Arg Gly Ile Gln Gln Leu Tyr Gly Ser Gln Ser Gly
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Ser Pro Thr Lys Met Pro Pro Pro Pro Arg Thr Thr Ser Arg Thr Phe
290 295 300

Ile Pro Asp Lys Pro Arg Asn Pro Thr Tyr Gly Pro Asn Ile Cys Asp
305 310 315 320

Gly Asn Phe Asp Thr Val Ala Val Leu Arg Gly Glu Met Phe Val Phe
325 330 335

Lys Glu Arg Trp Phe Trp Arg Val Arg Asn Asn Gln Val Met Asp Gly
340 345 350

Tyr Pro Met Pro Ile Gly Gln Phe Trp Arg Gly Leu Pro Ala Ser Ile
355 360 365

Asn Thr Ala Tyr Glu Arg Lys Asp Gly Lys Phe Val Phe Phe Lys Gly
370 375 380

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385 390 395 400

Lys His Ile Lys Glu Leu Gly Arg Gly Leu Pro Thr Asp Lys Ile Asp
405 410 415

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420 425 430

Asn Lys Tyr Tyr Arg Phe Asn Glu Glu Leu Arg Ala Val Asp Ser Glu
435 440 445

Tyr Pro Lys Asn Ile Lys Val Trp Glu Gly Ile Pro Glu Ser Pro Arg
450 455 460

Gly Ser Phe Met Gly Ser Asp Glu Val Phe Thr Tyr Phe Tyr Lys Gly
465 470 475 480

Asn Lys Tyr Trp Lys Phe Asn Asn Gln Lys Leu Lys Val Glu Pro Gly
485 490 495

Tyr Pro Lys Ser Ala Leu Arg Asp Trp Met Gly Cys Pro Ala Gly Gly
500 505 510

Arg Pro Asp Glu Gly Thr Glu Glu Glu Thr Glu Val Ile Ile Ile Glu
515 520 525

Val Asp Glu Glu Gly Ser Gly Ala Val Ser Ala Ala Ala Val Val Leu
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Pro Val Leu Leu Leu Leu Leu Val Leu Ala Val Gly Leu Ala Val Phe
545 550 555 560

Phe Phe Arg Arg His Gly Thr Pro Lys Arg Leu Leu Tyr Cys Gln Arg
565 570 575

Ser Leu Leu Asp Lys Val
580

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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Thr Leu Gly Thr Ala Leu Ala Ser Leu Gly Ser Ala Gln Ser Ser Ser
20 25 30

Phe Ser Pro Glu Ala Trp Leu Gln Gln Tyr Gly Tyr Leu Pro Pro Gly
35 40 45

Asp Leu Arg Thr His Thr Gln Arg Ser Pro Gln Ser Leu Ser Ala Ala
50 55 60

Ile Ala Ala Met Gln Lys Phe Tyr Gly Leu Gln Val Thr Gly Lys Ala
65 70 75 80

Asp Ala Asp Thr Met Lys Ala Met Arg Arg Pro Arg Cys Gly Val Pro
85 90 95

Asp Lys Phe Gly Ala Glu Ile Lys Ala Asn Val Arg Arg Lys Arg Tyr
100 105 110

Ala Ile Gln Gly Leu Lys Trp Gln His Asn Glu Ile Thr Phe Cys Ile
115 120 125

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130 135 140

Arg Lys Ala Phe Arg Val Trp Glu Ser Ala Thr Pro Leu Arg Phe Arg
145 150 155 160

Glu Val Pro Tyr Ala Tyr Ile Arg Glu Gly His Glu Lys Gln Ala Asp
165 170 175

Ile Met Ile Phe Phe Ala Glu Gly Phe His Gly Asp Ser Thr Pro Phe
180 185 190

Asp Gly Glu Gly Gly Phe Leu Ala His Ala Tyr Phe Pro Gly Pro Asn
195 200 205

Ile Gly Gly Asp Thr His Phe Asp Ser Ala Glu Pro Trp Thr Val Arg
210 215 220

Asn Glu Asp Leu Asn Gly Asn Asp Ile Phe Leu Val Ala Val His Glu
225 230 235 240

Leu Gly His Ala Leu Gly Leu Glu His Ser Ser Asp Pro Ser Ala Ile
245 250 255

Met Ala Pro Phe Tyr Gln Trp Met Asp Thr Glu Asn Phe Val Leu Pro
260 265 270

Asp Asp Asp Arg Arg Gly Ile Gln Gln Leu Tyr Gly Gly Glu Ser Gly
275 280 285

Phe Pro Thr Lys Met Pro Pro Gln Pro Arg Thr Thr Ser Arg Pro Ser
290 295 300

Val Pro Asp Lys Pro Lys Asn Pro Thr Tyr Gly Pro Asn Ile Cys Asp
305 310 315 320

Gly Asn Phe Asp Thr Val Ala Met Leu Arg Gly Glu Met Phe Val Phe
325 330 335

Lys Glu Arg Trp Phe Trp Arg Val Arg Asn Asn Gln Val Met Asp Gly
340 345 350

Tyr Pro Met Pro Ile Gly Gln Phe Trp Arg Gly Leu Pro Ala Ser Ile
355 360 365

Asn Thr Ala Tyr Glu Arg Lys Asp Gly Lys Phe Val Phe Phe Lys Gly
370 375 380

Asp Lys His Trp Val Phe Asp Glu Ala Ser Leu Glu Pro Gly Tyr Pro
385 390 395 400

Lys His Ile Lys Glu Leu Gly Arg Gly Leu Pro Thr Asp Lys Ile Asp
405 410 415

Ala Ala Leu Phe Trp Met Pro Asn Gly Lys Thr Tyr Phe Phe Arg Gly
420 425 430

Asn Lys Tyr Tyr Arg Phe Asn Glu Glu Leu Arg Ala Val Asp Ser Glu
435 440 445

Tyr Pro Lys Asn Ile Lys Val Trp Glu Gly Ile Pro Glu Ser Pro Arg
450 455 460

Gly Ser Phe Met Gly Ser Asp Glu Val Phe Thr Tyr Phe Tyr Lys Gly
465 470 475 480

Asn Lys Tyr Trp Lys Phe Asn Asn Gln Lys Leu Lys Val Glu Pro Gly
485 490 495

Tyr Pro Lys Ser Ala Leu Arg Asp Trp Met Gly Cys Pro Ser Gly Gly
500 505 510

Arg Pro Asp Glu Gly Thr Glu Glu Glu Thr Glu Val Ile Ile Ile Glu
515 520 525

Val Asp Glu Glu Gly Gly Gly Ala Val Ser Ala Ala Ala Val Val Leu
530 535 540

Pro Val Leu Leu Leu Leu Leu Val Leu Ala Val Gly Leu Ala Val Phe
545 550 555 560

Phe Phe Arg Arg His Gly Thr Pro Arg Arg Leu Leu Tyr Cys Gln Arg
565 570 575

Ser Leu Leu Asp Lys Val
580

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rattus rattus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Phe Ser Pro Glu Ala Trp Leu Gln Gln Tyr Gly Tyr Leu Pro Pro Gly
35 40 45

Asp Leu Arg Thr His Thr Gln Arg Ser Pro Gln Ser Leu Ser Ala Ala
50 55 60

Ile Ala Ala Ile Gln Arg Phe Tyr Gly Leu Gln Val Thr Gly Lys Ala
65 70 75 80

Asp Ser Asp Thr Met Lys Ala Met Arg Arg Pro Arg Cys Gly Val Pro
85 90 95

Asp Lys Phe Gly Thr Glu Ile Lys Ala Asn Val Arg Arg Lys Arg Tyr
100 105 110

Ala Ile Gln Gly Leu Lys Trp Gln His Asn Glu Ile Thr Phe Cys Ile
115 120 125

Gln Asn Tyr Thr Pro Lys Val Gly Glu Tyr Ala Thr Phe Glu Ala Ile
130 135 140

Arg Lys Ala Phe Arg Val Trp Glu Ser Ala Thr Pro Leu Arg Phe Arg
145 150 155 160

Glu Val Pro Tyr Ala Tyr Ile Arg Glu Gly His Glu Lys Gln Ala Asp
165 170 175

Ile Met Ile Leu Phe Ala Glu Gly Phe His Gly Asp Ser Thr Pro Phe
180 185 190

Asp Gly Glu Gly Gly Phe Leu Ala His Ala Tyr Phe Pro Gly Pro Asn
195 200 205

Ile Gly Gly Asp Thr His Phe Asp Ser Ala Glu Pro Trp Thr Val Gln
210 215 220

Asn Glu Asp Leu Asn Gly Asn Asp Ile Phe Leu Val Ala Val His Glu
225 230 235 240

Leu Gly His Ala Leu Gly Leu Glu His Ser Asn Asp Pro Ser Asp Ile
245 250 255

Met Ala Pro Phe Tyr Gln Trp Met Asp Thr Glu Asn Phe Val Leu Pro
260 265 270

Asp Asp Asp Arg Arg Gly Ile Gln Gln Leu Tyr Gly Ser Lys Ser Gly
275 280 285

Ser Pro Thr Lys Met Pro Pro Gln Pro Arg Thr Thr Ser Arg Pro Ser
290 295 300

Val Pro Asp Lys Pro Arg Asn Pro Thr Tyr Gly Pro Asn Ile Cys Asp
305 310 315 320

Gly Asn Phe Asp Thr Val Ala Met Leu Arg Gly Glu Met Phe Val Phe
325 330 335

Lys Glu Arg Trp Phe Trp Arg Val Arg Asn Asn Gln Val Met Asp Gly
340 345 350

Tyr Pro Met Pro Ile Gly Gln Phe Trp Arg Gly Leu Pro Ala Ser Ile
355 360 365

Asn Thr Ala Tyr Glu Arg Lys Asp Gly Lys Phe Val Phe Phe Lys Gly
370 375 380

Asp Lys His Trp Val Phe Asp Glu Ala Ser Leu Glu Pro Gly Tyr Pro
385 390 395 400

Lys His Ile Lys Glu Leu Gly Arg Gly Leu Pro Thr Asp Lys Ile Asp
405 410 415

Ala Ala Leu Phe Trp Met Pro Asn Gly Lys Thr Tyr Phe Phe Arg Gly
420 425 430

Asn Lys Tyr Tyr Arg Phe Asn Glu Glu Phe Arg Ala Val Asp Ser Glu
435 440 445

Tyr Pro Lys Asn Ile Lys Val Trp Glu Gly Ile Pro Glu Ser Pro Arg
450 455 460

Gly Ser Phe Met Gly Ser Asp Glu Val Phe Thr Tyr Phe Tyr Lys Gly
465 470 475 480

Asn Lys Tyr Trp Lys Phe Asn Asn Gln Lys Leu Lys Val Glu Pro Gly
485 490 495

Tyr Pro Lys Ser Ala Leu Arg Asp Trp Met Gly Cys Pro Ser Gly Gly
500 505 510

Arg Pro Asp Glu Gly Thr Glu Glu Glu Thr Glu Val Ile Ile Ile Glu
515 520 525

Val Asp Glu Glu Gly Ser Gly Ala Val Ser Ala Ala Ala Val Val Leu
530 535 540

Pro Val Leu Leu Leu Leu Leu Val Leu Ala Val Gly Leu Ala Val Phe
545 550 555 560

Phe Phe Arg Arg His Gly Thr Pro Lys Arg Leu Leu Tyr Cys Gln Arg
565 570 575

Ser Leu Leu Asp Lys Val
580

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus cookii

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Ser Pro Ala Pro Arg Pro Ser Arg Ser Leu Leu Leu Pro Leu Leu
1 5 10 15

Thr Leu Gly Thr Ala Leu Ala Ser Leu Gly Trp Ala Gln Gly Ser Asn
20 25 30

Phe Ser Pro Glu Ala Trp Leu Gln Gln Phe Gly Tyr Leu Pro Arg Gly
35 40 45

Asp Leu Arg Thr His Thr Gln Arg Ser Pro Gln Thr Leu Ser Val Asp
50 55 60

Ile Ala Ala Ile Gln Lys Phe Tyr Gly Leu Tyr Val Thr Gly Lys Ala
65 70 75 80

Tyr Ser Glu Thr Met Lys Ala Met Arg Arg Pro Arg Cys Gly Val Pro
85 90 95

Asp Lys Phe Gly Thr Glu Ile Lys Ala Asn Val Arg Arg Lys Arg Tyr
100 105 110

Ala Ile Gln Gly Leu Lys Trp Gln His Asn Glu Ile Thr Phe Cys Ile
115 120 125

Gln Asn Tyr Thr Pro Lys Val Gly Glu Tyr Ala Thr Phe Glu Ala Ile
130 135 140

Arg Lys Ala Phe Arg Val Trp Glu Ser Ala Thr Pro Leu Arg Phe Arg
145 150 155 160

Glu Val Pro Tyr Ala Tyr Ile Arg Glu Gly His Glu Lys Gln Ala Asp
165 170 175

Ile Met Ile Leu Phe Pro Glu Gly Leu His Gly Asp Ser Thr Pro Phe
180 185 190

Asp Gly Glu Gly Gly Phe Leu Ala His Ala Tyr Phe Pro Gly Pro Asn
195 200 205

Ile Gly Gly Asp Thr His Phe Asp Ser Ala Glu Pro Trp Thr Val Gln
210 215 220

Asn Glu Asp Leu Asn Gly Asn Asp Ile Phe Leu Val Ala Val His Glu
225 230 235 240

Leu Gly His Ala Leu Gly Leu Glu His Ser Asn Asp Pro Ser Asp Ile
245 250 255

Met Ser Pro Phe Tyr Gln Trp Met Asp Thr Glu Asn Phe Val Leu Pro
260 265 270

Asp Asp Asp Arg Arg Gly Ile Gln Gln Leu Tyr Gly Ser Lys Ser Gly
275 280 285

Ser Pro Thr Lys Met Pro Pro Gln Pro Arg Thr Thr Ser Arg Pro Ser
290 295 300

Val Pro Asp Lys Pro Lys Asn Pro Ala Tyr Gly Pro Asn Ile Cys Asp
305 310 315 320

Gly Asn Phe Asp Thr Val Ala Met Leu Arg Gly Glu Met Phe Val Phe
325 330 335

Lys Glu Arg Trp Leu Trp Arg Val Arg Asn Asn Gln Val Met Asp Gly
340 345 350

Tyr Pro Met Pro Ile Gly Gln Phe Trp Arg Gly Leu Pro Ala Ser Ile
355 360 365

Asn Thr Ala Tyr Glu Arg Lys Asp Gly Thr Phe Val Phe Phe Lys Gly
370 375 380

Asp Lys His Trp Val Cys Val Glu Ala Ser Leu Glu Pro Gly Tyr Ala
385 390 395 400

Asn His Ile Lys Glu Leu Val Arg Gly Leu Pro Ser Asp Lys Ile Asp
405 410 415

Thr Ala Leu Phe Trp Met Pro Asn Gly Lys Thr Tyr Phe Phe Arg Gly
420 425 430

Asn Lys Tyr Tyr Arg Phe Asn Glu Glu Phe Arg Ala Val Asp Ser Glu
435 440 445

Tyr Pro Lys Asn Ile Lys Val Trp Glu Gly Ile Pro Glu Ser Pro Arg
450 455 460

Gly Ser Phe Met Gly Ser Asp Glu Val Phe Thr Tyr Phe Tyr Lys Gly
465 470 475 480

Asn Lys Tyr Trp Lys Phe Asn Asn Gln Lys Leu Lys Val Glu Pro Gly
485 490 495

Tyr Pro Lys Ser Ala Leu Arg Asp Trp Met Gly Cys Pro Ser Gly Gly
500 505 510

Arg Pro Asp Glu Gly Thr Glu Glu Glu Thr Glu Val Ile Ile Ile Glu
515 520 525

Val Asp Glu Glu Gly Ser Gly Ala Val Ser Ala Ala Ala Val Val Leu
530 535 540

Pro Val Leu Leu Leu Leu Leu Val Leu Ala Val Gly Leu Ala Val Phe
545 550 555 560

Phe Phe Arg Arg His Gly Thr Pro Lys Arg Leu Leu Tyr Cys Gln Arg
565 570 575

Ser Leu Leu Asp Lys Val
580

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "X is Abz-G"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:6
(D) OTHER INFORMATION:/product= "X is Lnor"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:9
(D) OTHER INFORMATION:/product= "X is Y(NO2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Xaa Pro Leu Gly Leu Xaa Ala Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "X is Abz-S"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/product= "X is hydroxyproline"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:9
(D) OTHER INFORMATION:/product= "X is Y (NO2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Xaa Lys Tyr Pro Xaa Ala Leu Phe Xaa Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Cys Asp Gly Asn Phe Asp Thr Val Ala Met Leu Arg Gly Glu Met
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "X is Mca-P"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/product= "X is Dpa-A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Xaa Leu Gly Leu Xaa Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryctolagus cuniculus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGGGATCCCT GTGGGTCACT TCTTCT
26

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryctolagus cuniculus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCGCTCGAGC TGGCACCATT ACTAGC
26

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "X is K (Abz)-PEGA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Leu Phe Phe Xaa
1

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "X is Abz-G"

(ix) FEATURE:
(A) NAME/KEY: Cleavage-site
(B) LOCATION:4..5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Xaa Pro Leu Gly Leu Xaa Ala Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "X = J"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Tyr Pro Leu Xaa Met Lys Gly Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:2..6
(D) OTHER INFORMATION:/product= "each X =J"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Asn Xaa Tyr Pro Xaa Xaa Tyr Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:3..8
(D) OTHER INFORMATION:/product= "each X = J"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Tyr Pro Xaa Xaa Met Lys Gly Xaa Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TGGTATGTGG TCTGTGT
17

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TGTGGTTCAG TTGTGGT
17

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ACCACAACTG AACCACA
17

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGACTCATGG TGAGGAC
17

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGGATACAGG TGTCGGA
17

CLAIMS

1. The use of an agent in the manufacture ofa medicament forthe treatment of bone metabolic disease, characterised in that the agent acts by inhibitionof the production or action of a membrane associated protease or the matrix metalloprotease MMP-12 involved in the resorptive activity of osteoclasts.

2. The use claimed in Claim 1, wherein the agent acts by inhibition of the production or action of a membrane-type matrix metallo-proteinase (MT-MMP) or the matrix metalloproteinase MMP-12 involved in the resorptive activity of osteoclasts.

3. The use claimed in Claim 2, wherein a protease is inhibited which is involved in the recruitment, proliferation, differentiation, or migration of osteoclast precursor cells or in the migration, fusion, attachment , polarisation, activity in removal of mineralised osseous substance, or death of osteoclasts.

4. The use claimed in any preceding claim, wherein the agent is an antibody selectively immunoreactive with a said protease.

5. The use claimed in any one of Claims 1 to 3, wherein the agent is an antisense oligonucleotide or oligonucleotide analogue directed against a gene involved in the production of a said protease.

6. The use claimed in any one of Claims 1 to 3, wherein the agent is a protease substrate mimic inhibitor.

7. The use claimed in any one of Claims 1 to 3, wherein the agent is a broad spectrum matrix metalloproteinase (MMP) inhibitor or a broad spectrum membrane-associated metallo-proteinase inhibitor.

8. The use claimed in any one of Claims 1 to 3, wherein the agent is a selective inhibitor of MT1-MMP, MMP-12 or a specific member of one of the families of membrane-associated metalloproteinase, such as the meltrins or ADAMs.

9. The use claimed in any one of Claims 1 to 3, wherein the agent is a peptide or peptide analogue obtained by screening a peptide library for peptides reactive with a said protease.

10. The use of an agent in the manufacture of a medicament for the treatment of bone metabolic disease by inhibition of the recruitment, proliferation, differentiation, or migration of osteoclast precursor cells or in the migration, fusion, attachment, polarisation, or death of osteoclasts.

11. The use claimed in Claim 10, wherein said agent produces said inhibition by inhibiting the production or action of a proteinase.

12. An anti-bone resorption agent comprising a proteinase inhibitor active against a proteinase involved in bone resorption operatively linked to a ligand having binding specificity targeting the inhibitor to said proteinase or to the environment of the proteinase.

Fig 1
Sheet a

```
CCG CTA GGA ATC CAA AGT CGG TGC CTC CGG AAG ACA AAG GCG CCC CCG AGG GAG   54
TGG CGG CGC GAC CCC TAG GCG AGG GCC CCG CCG CGG AAC CGC CCA GCC CGG CTG  108
CCC CGA CGG TCG CGG ACC ATG TCT CCC GCC CCA CGA CCC TCC CGC AGG CTC CTG  162
                        Met Ser Pro Ala Pro Arg Pro Ser Arg Arg Leu Leu   12

CTC CCC CTG CTC ACA CTC GGC ACC GCA CTC GCC TCC CTC GGC TCG GCC AAA AGC  216
Leu Pro Leu Leu Thr Leu Gly Thr Ala Leu Ala Ser Leu Gly Ser Ala Lys Ser   30

AAC AGC TTC AGC CCC GAA GCC TGG CTG CAG CAG TAT GGC TAC CTG CCT CCA GGG  270
Asn Ser Phe Ser Pro Glu Ala Trp Leu Gln Gln Tyr Gly Tyr Leu Pro Pro Gly   48

GAC CTA CGC ACC CAC ACA CAG CGC TCT CCT CAG TCA CTG TCA GCT GCC ATT GCT  324
Asp Leu Arg Thr His Thr Gln Arg Ser Pro Gln Ser Leu Ser Ala Ala Ile Ala   66

GCC ATG CAG AGG TTC TAC GGT TTG CGA GTG ACA GGC AAG GCC GAT ACA GAC ACC  378
Ala Met Gln Arg Phe Tyr Gly Leu Arg Val Thr Gly Lys Ala Asp Thr Asp Thr   84

ATG AAG GCC ATG AGG CGC CCC CGC TGC GGT GTT CCA GAC AAG TTT GGG GCT GAG  432
Met Lys Ala Met Arg Arg Pro Arg Cys Gly Val Pro Asp Lys Phe Gly Ala Glu  102

ATC AAG GCC AAT GTC CGA AGG AAG CGC TAC GCC ATC CAG GGC CTC AAA TGG CAG  486
Ile Lys Ala Asn Val Arg Arg Lys Arg Tyr Ala Ile Gln Gly Leu Lys Trp Gln  120

CAT AAT GAG ATC ACT TTC TGC ATC CAG AAT TAC ACC CCC AAG GTG GGC GAA TAT  540
His Asn Glu Ile Thr Phe Cys Ile Gln Asn Tyr Thr Pro Lys Val Gly Glu Tyr  138

GCC ACA TTC GAG GCC ATT CGC AAG GCA TTC CGC GTG TGG GAG AGC GCC ACA CCG  594
Ala Thr Phe Glu Ala Ile Arg Lys Ala Phe Arg Val Trp Glu Ser Ala Thr Pro  156

CTG CGC TTC CGC GAG GTG CAC TAT GCC TAC ATC CGC GAT GGC CGT GAG AAG CAG  648
Leu Arg Phe Arg Glu Val His Tyr Ala Tyr Ile Arg Asp Gly Arg Glu Lys Gln  174
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Fig 1

Sheet b

```
GCC GAC ATC ATG ATC TTC TTT GCC GAG GGC TTC CAT GGC GAC AGC ACG CCC TTC  702
Ala Asp Ile Met Ile Phe Phe Ala Glu Gly Phe His Gly Asp Ser Thr Pro Phe  192

GAT GGC GAG GGT GGC TTC CTG GCC CAC GCC TAC TTC CCG GGC CCC AAC ATT GGA  756
Asp Gly Glu Gly Gly Phe Leu Ala His Ala Tyr Phe Pro Gly Pro Asn Ile Gly  210

GGG GAC ACC CAC TTT GAC TCC GCG GAG CCC TGG ACT GTC CGG AAT GAG GAC CTG  810
Gly Asp Thr His Phe Asp Ser Ala Glu Pro Trp Thr Val Arg Asn Glu Asp Leu  228

AAC GGG AAT GAC ATC TTC CTG GTG GCT GTG CAT GAG CTG GGC CAT GCC CTG GGC  864
Asn Gly Asn Asp Ile Phe Leu Val Ala Val His Glu Leu Gly His Ala Leu Gly  246

CTG GAG CAC TCC AAT GAC CCC TCA GCC ATC ATG GCA CCG TTT TAC CAA TGG ATG  918
Leu Glu His Ser Asn Asp Pro Ser Ala Ile Met Ala Pro Phe Tyr Gln Trp Met  264

GAC ACA GAG AAC TTC GTG CTG CCT GAT GAT GAC CGC CGG GGC ATC CAA CAG CTT  972
Asp Thr Glu Asn Phe Val Leu Pro Asp Asp Asp Arg Arg Gly Ile Gln Gln Leu  282

TAT GGG AGC CAG TCG GGG TCC CCC ACA AAG ATG CCT CCT CCA CCC AGG ACA ACC 1026
Tyr Gly Ser Gln Ser Gly Ser Pro Thr Lys Met Pro Pro Pro Pro Arg Thr Thr  300

TCC CGG ACT TTT ATC CCC GAT AAG CCC AGG AAC CCC ACC TAC GGG CCC AAC ATC 1080
Ser Arg Thr Phe Ile Pro Asp Lys Pro Arg Asn Pro Thr Tyr Gly Pro Asn Ile  318

TGT GAC GGG AAC TTT GAC ACT GTG GCC GTG CTC CGA GGA GAG ATG TTT GTC TTC 1134
Cys Asp Gly Asn Phe Asp Thr Val Ala Val Leu Arg Gly Glu Met Phe Val Phe  336

AAG GAG CGC TGG TTC TGG AGG GTG AGG AAC AAC CAA GTG ATG GAC GGC TAC CCA 1188
Lys Glu Arg Trp Phe Trp Arg Val Arg Asn Asn Gln Val Met Asp Gly Tyr Pro  354
```

Figure 1

Sheet c

```
ATG CCC ATC GGC CAG TTC TGG CGG GGC CTG CCT GCT TCC ATC AAC ACC GCC TAC  1242
Met Pro Ile Gly Gln Phe Trp Arg Gly Leu Pro Ala Ser Ile Asn Thr Ala Tyr  372

GAG AGG AAG GAT GGC AAA TTC GTC TTC TTC AAA GGA GAT AAG CAC TGG GTG TTT  1296
Glu Arg Lys Asp Gly Lys Phe Val Phe Phe Lys Gly Asp Lys His Trp Val Phe  390

GAC GAG GCT TCC CTG GAG CCT GGC TAC CCC AAG CAC ATC AAG GAG CTG GGC CGA  1350
Asp Glu Ala Ser Leu Glu Pro Gly Tyr Pro Lys His Ile Lys Glu Leu Gly Arg  408

GGG CTT CCC ACC GAC AAG ATC GAT GCC GCT CTC TTC TGG ATG CCC AAT GGA AAG  1404
Gly Leu Pro Thr Asp Lys Ile Asp Ala Ala Leu Phe Trp Met Pro Asn Gly Lys  426

ACC TAC TTC TTC CGG GGA AAC AAG TAC TAC CGA TTC AAC GAG GAG CTC AGG GCA  1458
Thr Tyr Phe Phe Arg Gly Asn Lys Tyr Tyr Arg Phe Asn Glu Glu Leu Arg Ala  444

GTG GAC AGC GAG TAC CCC AAG AAC ATC AAA GTG TGG GAA GGC ATC CCC GAG TCT  1512
Val Asp Ser Glu Tyr Pro Lys Asn Ile Lys Val Trp Glu Gly Ile Pro Glu Ser  462

CCC AGA GGG TCG TTC ATG GGC AGT GAT GAA GTC TTC ACT TAC TTC TAC AAG GGG  1566
Pro Arg Gly Ser Phe Met Gly Ser Asp Glu Val Phe Thr Tyr Phe Tyr Lys Gly  480

AAC AAA TAC TGG AAA TTC AAC AAC CAG AAG CTG AAG GTG GAG CCC GGC TAC CCC  1620
Asn Lys Tyr Trp Lys Phe Asn Asn Gln Lys Leu Lys Val Glu Pro Gly Tyr Pro  498

AAG TCC GCC CTG CGG GAC TGG ATG GGC TGC CCG GCT GGG GGC CGT CCG GAT GAG  1674
Lys Ser Ala Leu Arg Asp Trp Met Gly Cys Pro Ala Gly Gly Arg Pro Asp Glu  516

GGG ACT GAG GAA GAG ACG GAG GTG ATC ATC ATC GAG GTG GAC GAG GAG GGC AGC  1728
Gly Thr Glu Glu Glu Thr Glu Val Ile Ile Ile Glu Val Asp Glu Glu Gly Ser  534
```

Figure 1

Sheet d

```
GGA GCC GTG AGC GCG GCC GCC GTG GTG CTG CCC GTG CTG CTG CTA CTC CTG GTG  1782
Gly Ala Val Ser Ala Ala Ala Val Val Leu Pro Val Leu Leu Leu Leu Leu Val  552

CTG GCC GTG GGC CTG GCG GTC TTC TTC TTC AGG CGC CAC GGG ACT CCG AAG CGA  1836
Leu Ala Val Gly Leu Ala Val Phe Phe Phe Arg Arg His Gly Thr Pro Lys Arg  570

CTG CTC TAC TGC CAG CGT TCC CTG CTG GAC AAG GTC TGA CCC CCA CCG CTG GCC  1890
Leu Leu Tyr Cys Gln Arg Ser Leu Leu Asp Lys Val *                        592

CAC CCA CTC CCA CCG CAA GGA CTT TGC TCT TCC GAT TGT ATC CAA TAA AAA ATA  1944
GCA TCA GCA AAA AAA AAA AAA AAA AAA A                                    1972
```

Fig 2
Sheet a

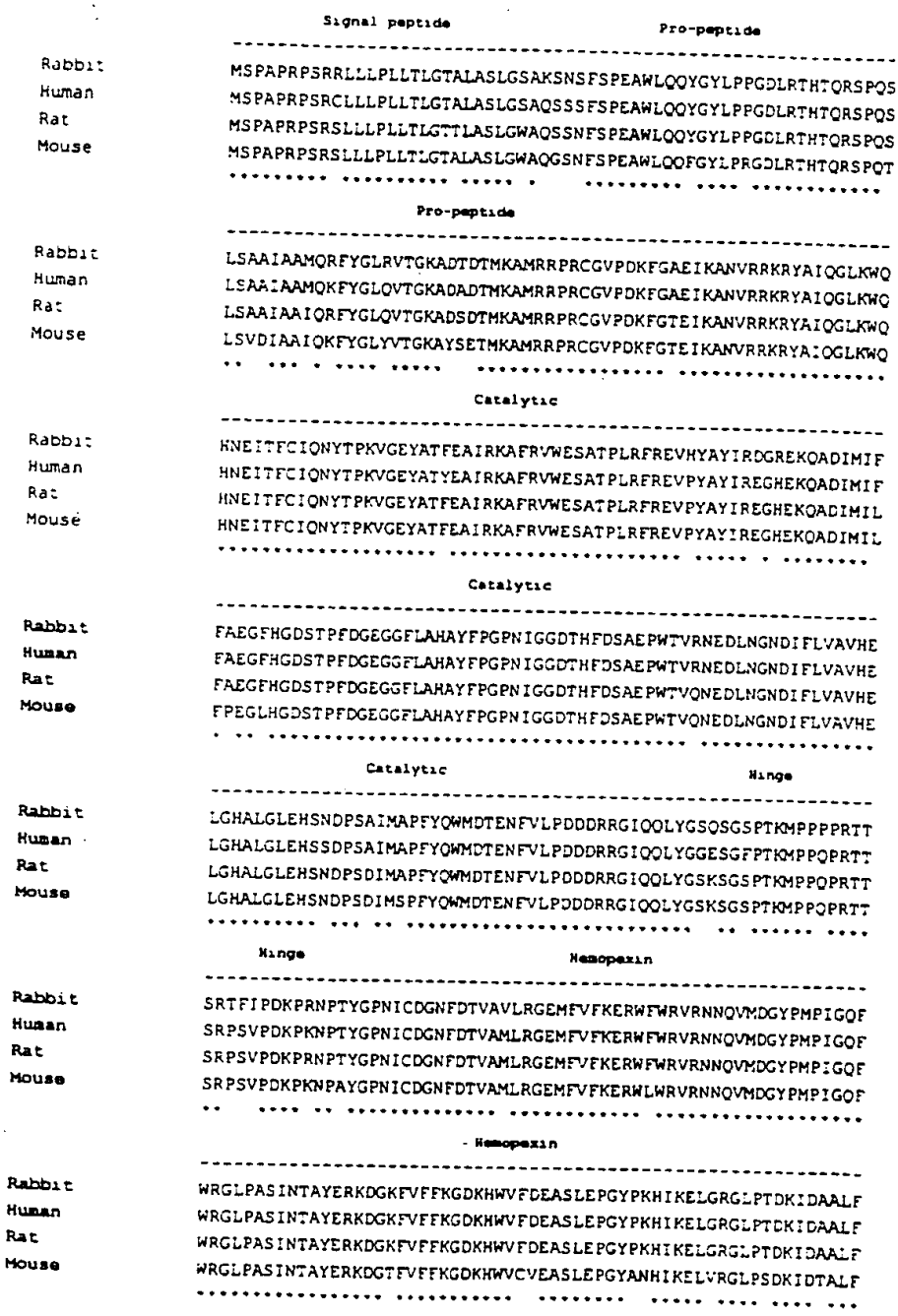

Signal peptide
Pro-peptide
Rabbit MSPAPRPSRRLLLPLLTLGTALASLGSAKSNSFSPEAWLQQYGYLPPGDLRTHTQRSPQS
Human MSPAPRPSRCLLLPLLTLGTALASLGSAQSSSFSPEAWLQQYGYLPPGDLRTHTQRSPQS
Rat MSPAPRPSRSLLLPLLTLGTTLASLGWAQSSNFSPEAWLQQYGYLPPGDLRTHTQRSPQS
Mouse MSPAPRPSRSLLLPLLTLGTALASLGWAQGSNFSPEAWLQQFGYLPRGDLRTHTQRSPQT
Pro-peptide
Rabbit LSAAIAAMQRFYGLRVTGKADTDTMKAMRRPRCGVPDKFGAEIKANVRRKRYAIQGLKWQ
Human LSAAIAAMQKFYGLQVTGKADADTMKAMRRPRCGVPDKFGAEIKANVRRKRYAIQGLKWQ
Rat LSAAIAAIQRFYGLQVTGKADSDTMKAMRRPRCGVPDKFGTEIKANVRRKRYAIQGLKWQ
Mouse LSVDIAAIQKFYGLYVTGKAYSETMKAMRRPRCGVPDKFGTEIKANVRRKRYAIQGLKWQ
Catalytic
Rabbit HNEITFCIQNYTPKVGEYATFEAIRKAFRVWESATPLRFREVHYAYIRDGREKQADIMIF
Human HNEITFCIQNYTPKVGEYATYEAIRKAFRVWESATPLRFREVPYAYIREGHEKQADIMIF
Rat HNEITFCIQNYTPKVGEYATFEAIRKAFRVWESATPLRFREVPYAYIREGHEKQADIMIL
Mouse HNEITFCIQNYTPKVGEYATFEAIRKAFRVWESATPLRFREVPYAYIREGHEKQADIMIL
Catalytic
Rabbit FAEGFHGDSTPFDGEGGFLAHAYFPGPNIGGDTHFDSAEPWTVRNEDLNGNDIFLVAVHE
Human FAEGFHGDSTPFDGEGGFLAHAYFPGPNIGGDTHFDSAEPWTVRNEDLNGNDIFLVAVHE
Rat FAEGFHGDSTPFDGEGGFLAHAYFPGPNIGGDTHFDSAEPWTVQNEDLNGNDIFLVAVHE
Mouse FPEGLHGDSTPFDGEGGFLAHAYFPGPNIGGDTHFDSAEPWTVQNEDLNGNDIFLVAVHE
Catalytic
Hinge
Rabbit LGHALGLEHSNDPSAIMAPFYQWMDTENFVLPDDDRRGIQQLYGSQSGSPTKMPPPRTT
Human LGHALGLEHSSDPSAIMAPFYQWMDTENFVLPDDDRRGIQQLYGGESGFPTKMPPQPRTT
Rat LGHALGLEHSNDPSDIMAPFYQWMDTENFVLPDDDRRGIQQLYGSKSGSPTKMPPQPRTT
Mouse LGHALGLEHSNDPSDIMSPFYQWMDTENFVLPDDDRRGIQQLYGSKSGSPTKMPPQPRTT
Hinge
Hemopexin
Rabbit SRTFIPDKPRNPTYGPNICDGNFDTVAVLRGEMFVFKERWFWRVRNNQVMDGYPMPIGQF
Human SRPSVPDKPKNPTYGPNICDGNFDTVAMLRGEMFVFKERWFWRVRNNQVMDGYPMPIGQF
Rat SRPSVPDKPRNPTYGPNICDGNFDTVAMLRGEMFVFKERWFWRVRNNQVMDGYPMPIGQF
Mouse SRPSVPDKPKNPAYGPNICDGNFDTVAMLRGEMFVFKERWLWRVRNNQVMDGYPMPIGQF
Hemopexin
Rabbit WRGLPASINTAYERKDGKFVFFKGDKHWVFDEASLEPGYPKHIKELGRGLPTDKIDAALF
Human WRGLPASINTAYERKDGKFVFFKGDKHWVFDEASLEPGYPKHIKELGRGLPTDKIDAALF
Rat WRGLPASINTAYERKDGKFVFFKGDKHWVFDEASLEPGYPKHIKELGRGLPTDKIDAALF
Mouse WRGLPASINTAYERKDGTFVFFKGDKHWVCVEASLEPGYANHIKELVRGLPSDKIDTALF

Fig 2
Sheet b

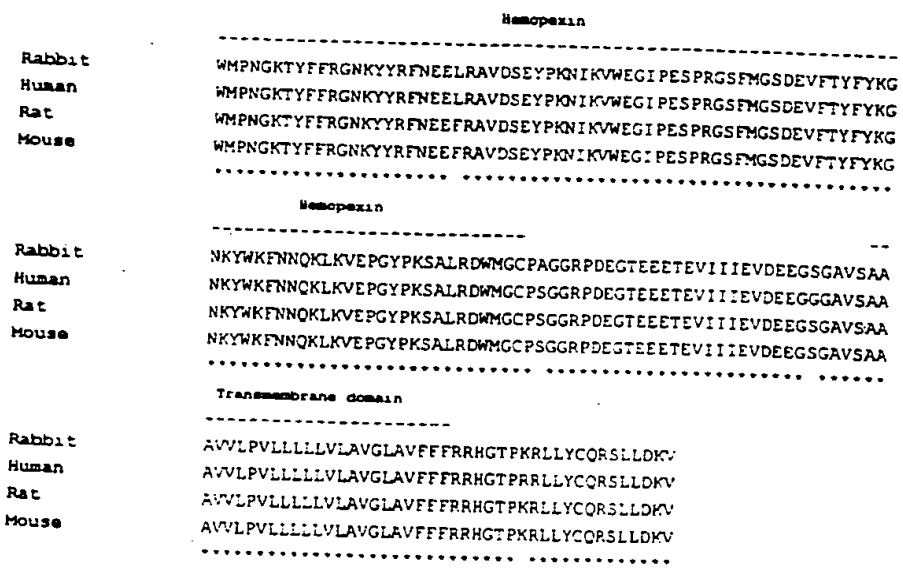

Hemopexin
Rabbit
Human
Rat
Mouse
Hemopexin
Rabbit
Human
Rat
Mouse
Transmembrane domain
Rabbit
Human
Rat
Mouse

Fig 3

Constructs for expression of rabbit MT1-MMP in E.coli:

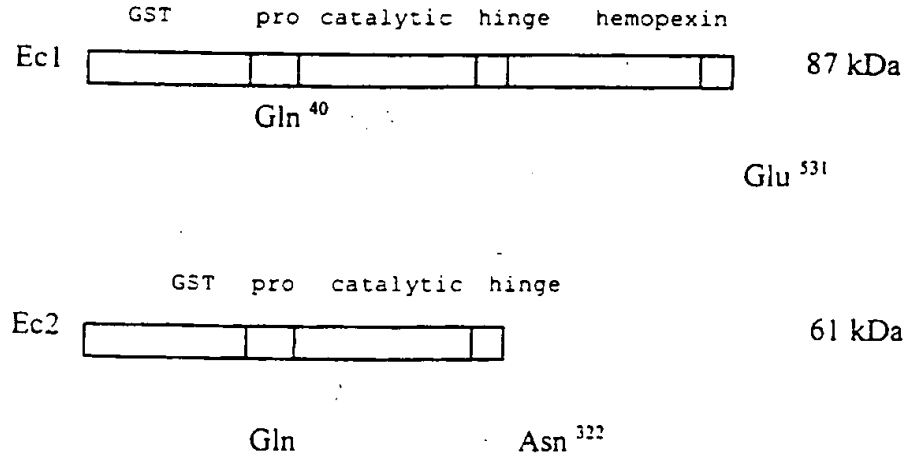


Figure 4
Sheet a

```
CTGAATTGAACCATTCAGGAGAAGTTCGCAATGAAGTTTCTTCTATTGATACTGACCCTG   60
      END                          M  K  F  L  L  L  I  L  T  L    10

TGGGTCACTTCTTCTGGAGCTGATCCTCTGAAGGAAAACGATATGCTATTTGCTGAAAAC  120
W  V  T  S  S  G  A  D  P  L  K  E  N  D  M  L  F  A  E  N     30

TACTTGGAAAACTTTTATGGCCTTAAGGTGGAGAGAATTCCAATGACAAAAATGAAAACT  180
Y  L  E  N  F  Y  G  L  K  V  E  R  I  P  M  T  K  M  K  T     50

AACAGGAACTTCATAGAGGAAAAAGTCCAGGAAATGCAGCAATTCTTGGGGCTAAATGTG  240
N  R  N  F  I  E  E  K  V  Q  E  M  Q  Q  F  L  G  L  N  V     70
                                                   ↑

ACTGGGCAACTGGACACATCTACTTTGGAAATGATGCACAAGCCTCGATGTGGAGTGCCT  300
T  G  Q  L  D  T  S  T  L  E  M  M  H  K  P  R  C  G  V  P     90

GATGTTTATCATTTCAAAACCATGCCAGGGAGACCAGTATGGAGGAAACATTACATCACC  360
D  V  Y  H  F  K  T  M  P  G  R  P  V  W  R  K  H  Y  I  T    110

TACAGAATCAAAAATTACACTCCAGACATGAAGCGTGAGGATGTTGAGTATGCCATTCAG  420
Y  R  I  K  N  Y  T  P  D  M  K  R  E  D  V  E  Y  A  I  Q    130
          *

AAAGCTTTTCAAGTATGGAGCGATGTGACCCCCCTGAAATTCAGAAAGATTACGACAGGC  480
K  A  F  Q  V  W  S  D  V  T  P  L  K  F  R  K  I  T  T  G    150

AAGGCTGACATCATGATACTTTTTGCTAGTGGAGCTCATGGAGACTATGGTGCTTTTGAT  540
K  A  D  I  M  I  L  F  A  S  G  A  H  G  D  Y  G  A  F  D    170

GGCAGAGGTGGTGTCATAGCCCATGCTTTTGGGCCTGGACCTGGTATTGGAGGAGATACA  600
G  R  G  G  V  I  A  H  A  F  G  P  G  P  G  I  G  G  D  T    190

CATTTTGATGAGGATGAAATCTGGAGTAAAAGTTATAAAGGCACAAACTTGTTCCTTGTT  660
H  F  D  E  D  E  I  W  S  K  S  Y  K  G  T  N  L  F  L  V    210

GCTGTCCATGAGCTTGGCCATGCCTTGGGACTTGATCATTCAAATGATCCAAAGGCCATA  720
A  V  H  E  L  G  H  A  L  G  L  D  H  S  N  D  P  K  A  I    230

ATGTTTCCCACCTATGGTTATATTGATCTCAACACATTTCACCTCTCTGCTGATGACATA  780
M  F  P  T  Y  G  Y  I  D  L  N  T  F  H  L  S  A  D  D  I    250

CGTGGCATTCAGTCCCTTTATGGAGGCCCAGAGCAGCATCAACCCATGCCAAAACCTGAC  840
R  G  I  Q  S  L  Y  G  G  P  E  Q  H  Q  P  M  P  K  P  D    270

AATCCGGAACCAACTGCCTGTGACCACAATTTGAAATTTGATGCAGTTACTACAGTGGGA  900
N  P  E  P  T  A  C  D  H  N  L  K  F  D  A  V  T  T  V  G    290

AATAAAATATTTTTCTTTAAAGACAGCTTTTTCTGGTGGAAGATTCCTAAGAGTTCAACG  960
N  K  I  F  F  F  K  D  S  F  F  W  W  K  I  P  K  S  S  T    310

ACCAGTGTCCGTTTAATTTCTTCCTTATGGCCAACCTTGCCTTCAGGCATTGAGGCTGCT 1020
T  S  V  R  L  I  S  S  L  W  P  T  L  P  S  G  I  E  A  A    330

TATGAAATTGGAGACAGACATCAAGTATTCCTTTTTAAAGGTGACAAGTTCTGGTTAATT 1080
Y  E  I  G  D  R  H  Q  V  F  L  F  K  G  D  K  F  W  L  I    350

AGCCATCTAAGACTACAACCAAACTATCCCAAGAGCATACATTCCCTGGGCTTCCCTGAC 1140
S  H  L  R  L  Q  P  N  Y  P  K  S  I  H  S  L  G  F  P  D    370

TTTGTGAAAAAAATTGATGCAGCTGTCTTTAACCCCAGTCTCCGGAAGACCTACTTCTTT 1200
F  V  K  K  I  D  A  A  V  F  N  P  S  L  R  K  T  Y  F  F    390
                              ↑

GTGGATAATCTGTACTGGAGATACGATGAAAGGAGAGAGGTCATGGATGCTGGTTATCCC 1260
V  D  N  L  Y  W  R  Y  D  E  R  R  E  V  M  D  A  G  Y  P    410
```

Figure 4
Sheet b

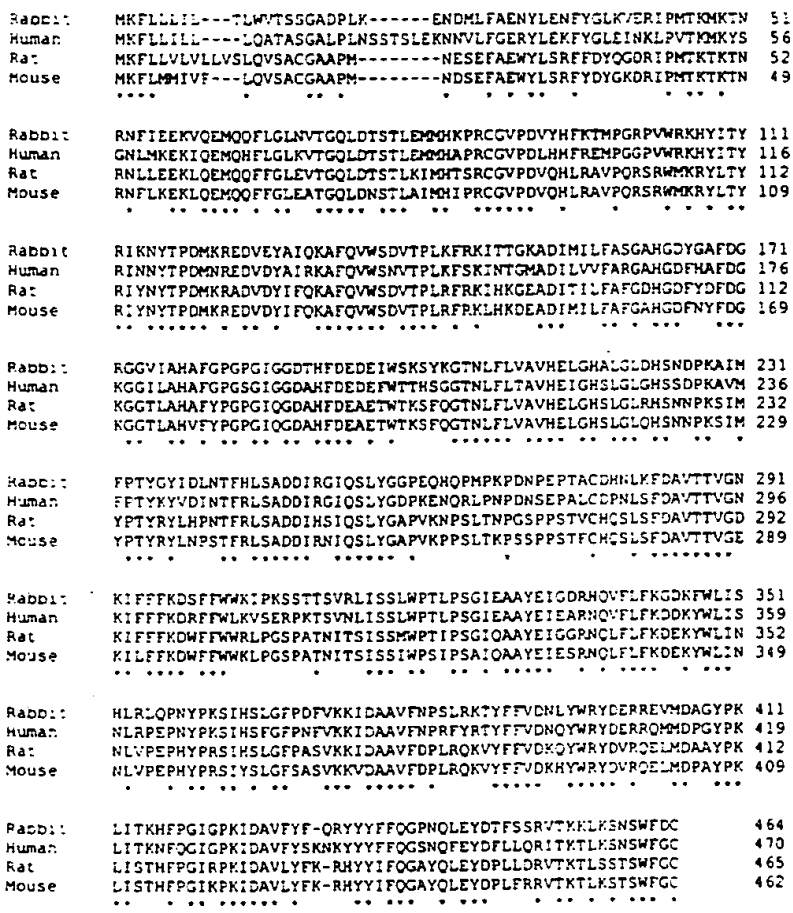
```
AAGCTGATCACCAAGCACTTCCCAGGAATTGGGCCGAAAATTGACGCAGTCTTCTATTTC 1320
K  L  I  T  K  H  F  P  G  I  G  P  K  I  D  A  V  F  Y  F    430

CAAAGATACTACTATTTCTTCCAGGGACCTAACCAACTTGAATATGACACATTTTCCAGT 1380
Q  R  Y  Y  Y  F  F  Q  G  P  N  Q  L  E  Y  D  T  F  S  S    450

CGTGTCACCAAGAAGCTGAAAAGCAATAGCTGGTTTGATTGCTAGTAATGGTGCCAGTTG 1440
R  V  T  K  K  L  K  S  N  S  W  F  D  C  *                  464

ACTTCCACTTAATAAGTATTTATTGCATACATACTATGTGATCAATGTAACACTACATGG 1500
TGATGTGTATCATAAAATAAAGTAAAATATATAGATCATAGAGAAGTGATTGTACCAAAT 1560
ATATAAGTTTTTCAATTTTGAAAACCCTTATTGTACATTTTTGCTTAACTCTACTATTAA 1620
ATTTGGAAATAGATGCTTTCAGAGGCCAAGAGAGTATCTTTTGTAGAATGCTTTGTGAGT 1680
TGGTTTCTACCATTGTTTGAGAAGTTACAATTATATATTATTCAAATAAAAAATTCAAAT 1740
AAATTATATATTATTCAAATAAAAACTTTGAAGAAAAAAAAAAAAAAAAAAA          1792
```

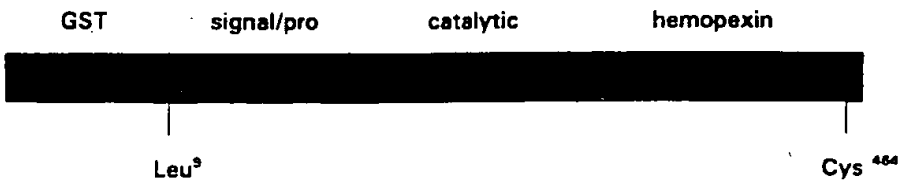
Figure 5



Alignment of amino acid sequences of rabbit, human, rat and mouse MMP-12.

Figure 6

Construct for expression of recombinant rabbit MMP-12 in E. Coli:



Predicted size of fusion protein: 83 kDa

Figure 7

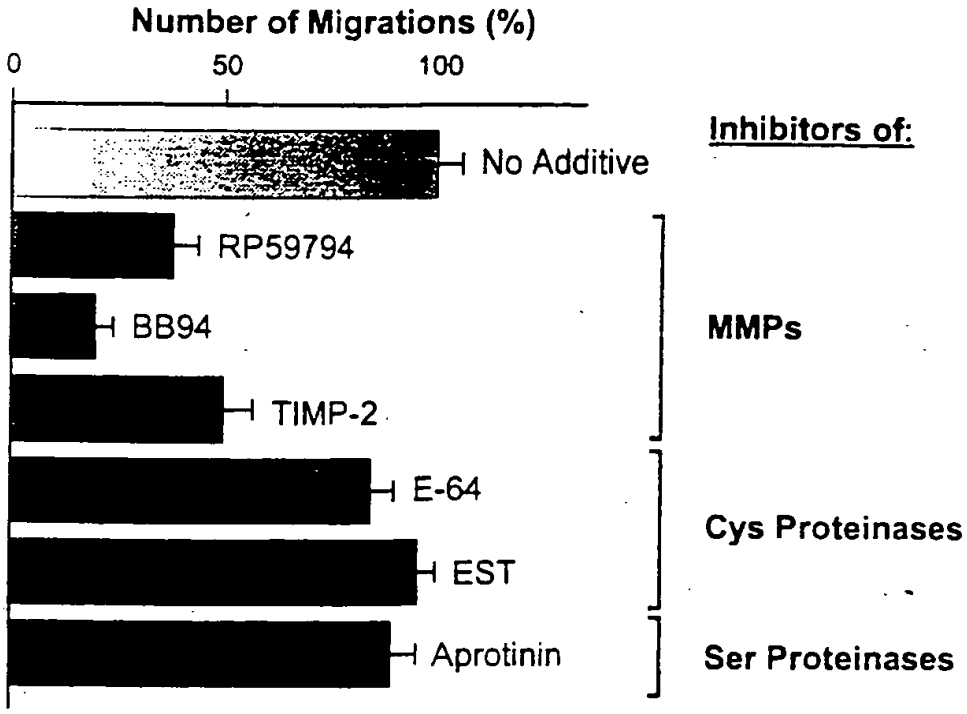
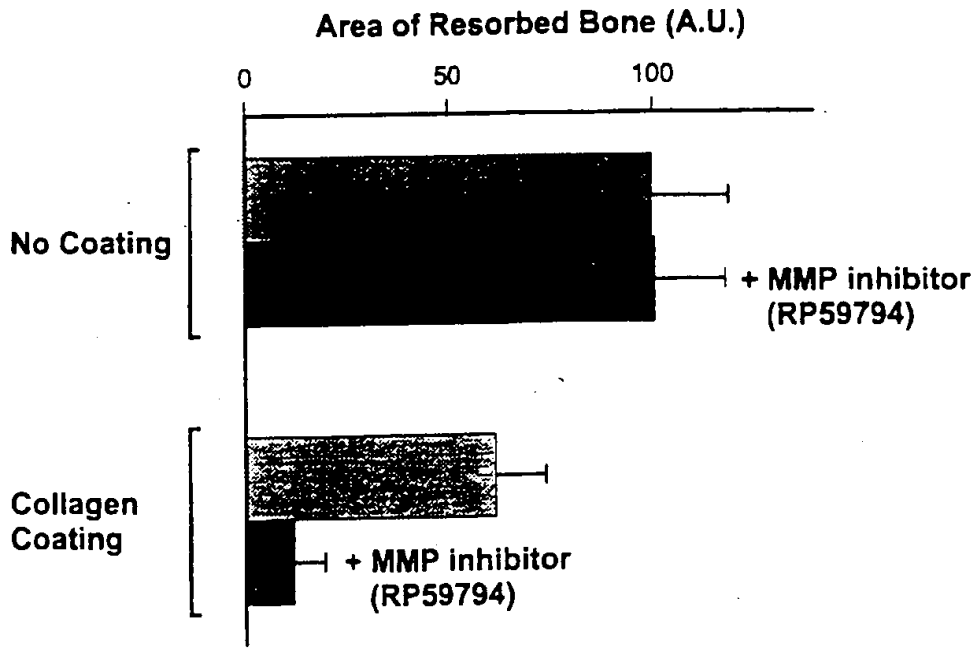

Number of Migrations (%)
0
50
100
No Additive
RP59794
BB94
TIMP-2
E-64
EST
Aprotinin
Inhibitors of:
MMPs
Cys Proteinases
Ser Proteinases

Figure 8


Area of Resorbed Bone (A.U.)
0
50
100
No Coating
+ MMP inhibitor (RP59794)
Collagen Coating
+ MMP inhibitor (RP59794)

Reduction of catlytic activity of MMP-9
by anti-MMP-9 and control antisera

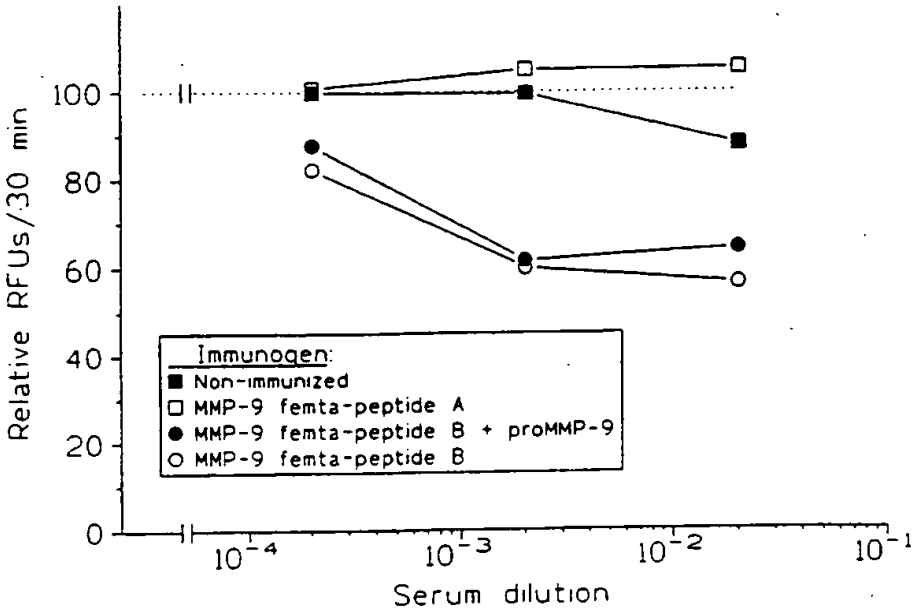
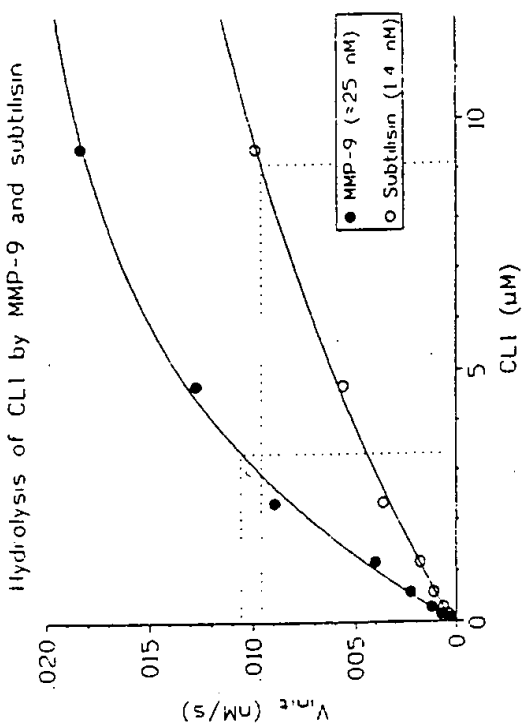
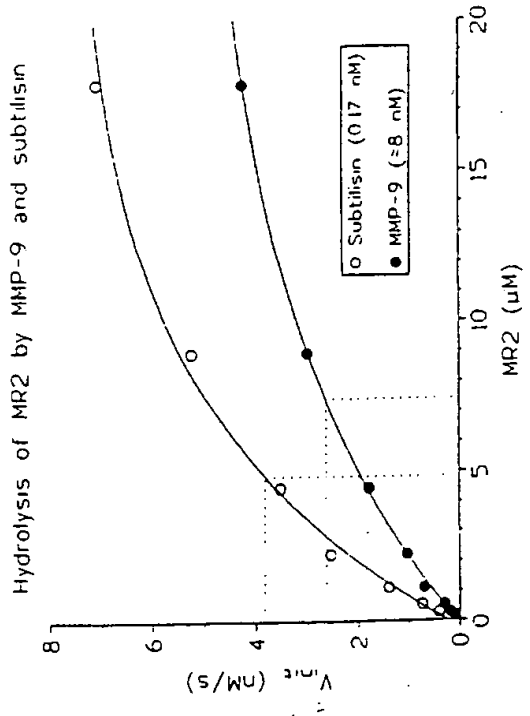


Figure 9



Graph B



Graph A

Figure 10

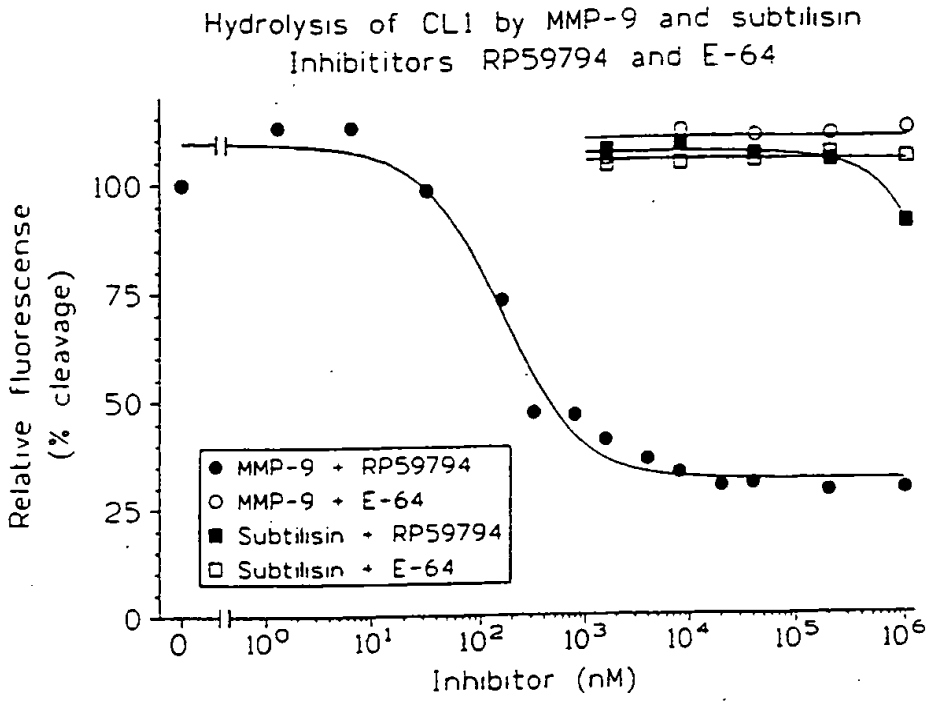

Hydrolysis of CL1 by MMP-9 and subtilisin
Inhibititors RP59794 and E-64
Relative fluorescense
(% cleavage)
100
75
50
25
0
MMP-9 + RP59794
MMP-9 + E-64
Subtilisin + RP59794
Subtilisin + E-64
0
10^0
10^1
10^2
10^3
10^4
10^5
10^6
Inhibitor (nM)

Figure 11

Figure 12

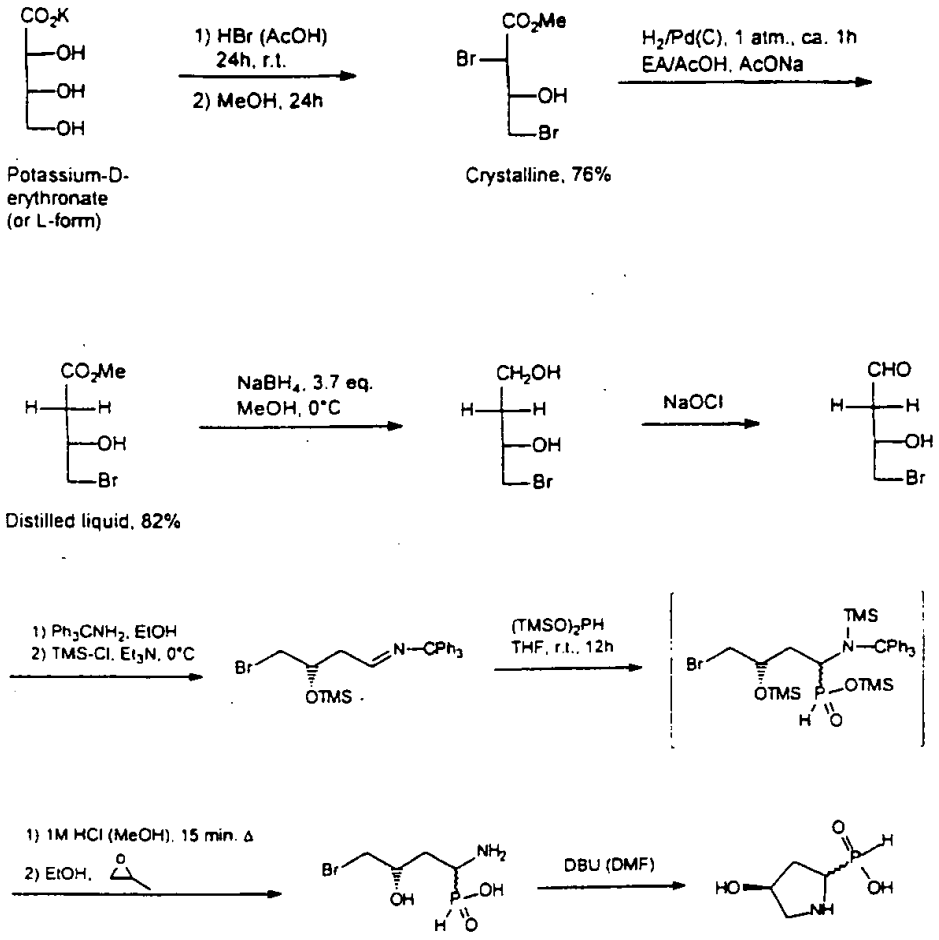

CO2K
OH
OH
OH
1) HBr (AcOH)
24h, r.t.
2) MeOH, 24h
CO2Me
Br
OH
Br
H2/Pd(C), 1 atm., ca. 1h
EA/AcOH, AcONa
Potassium-D-erythronate (or L-form)
Crystalline, 76%
CO2Me
H
H
OH
Br
NaBH4, 3.7 eq.
MeOH, 0°C
CH2OH
NaOCl
CHO
Distilled liquid, 82%
1) Ph3CNH2, EtOH
2) TMS-Cl, Et3N, 0°C
N—CPh3
OTMS
(TMSO)2PH
THF, r.t., 12h
TMS
OTMS
OTMS
1) 1M HCl (MeOH), 15 min. Δ
2) EtOH,
NH2
DBU (DMF)
HO
NH

Figure13

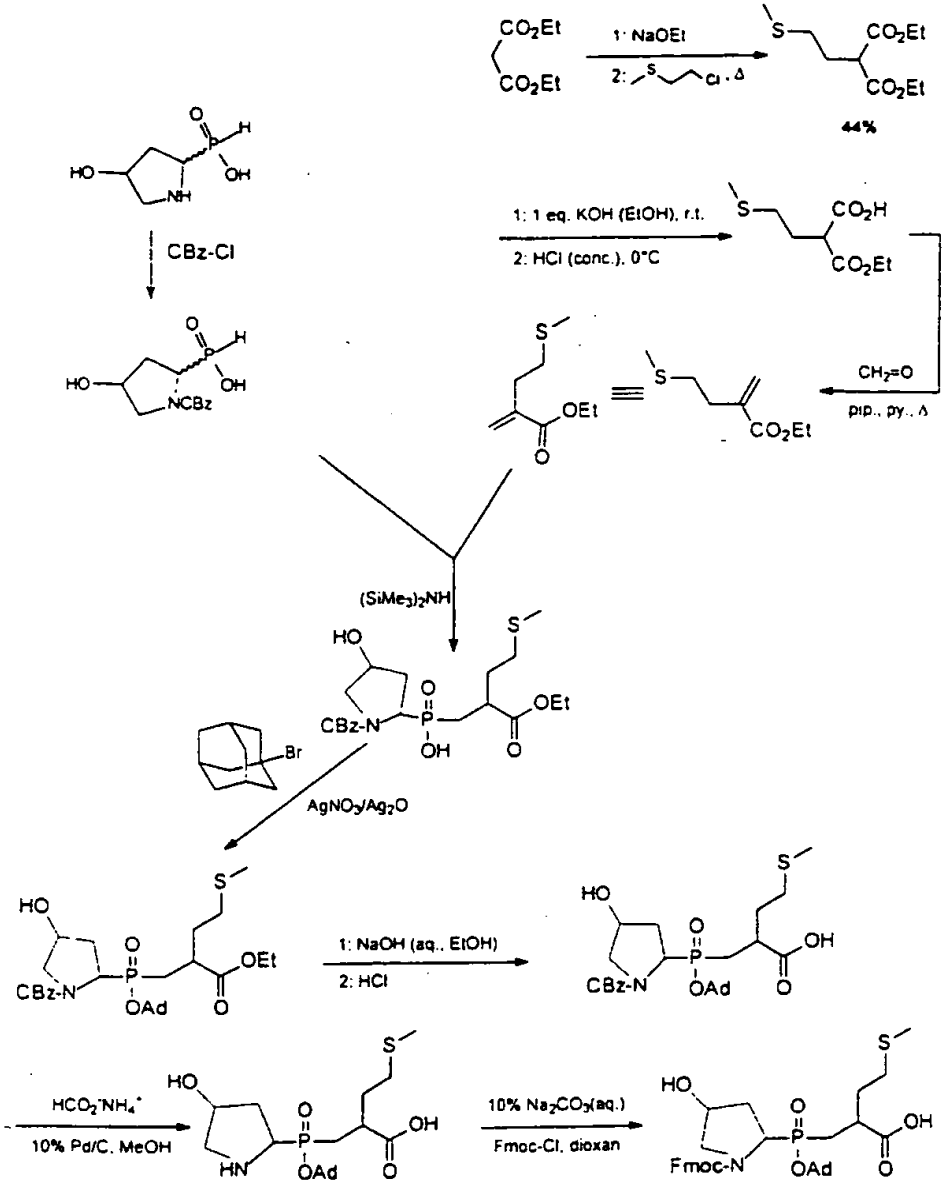

1: NaOEt
2:
44%
CBz-Cl
1: 1 eq. KOH (EtOH), r.t.
2: HCl (conc.), 0°C
CH2=O
pip., py., Δ
(SiMe3)2NH
AgNO3/Ag2O
1: NaOH (aq., EtOH)
2: HCl
HCO2-NH4+
10% Pd/C, MeOH
10% Na2CO3(aq.)
Fmoc-Cl, dioxan

Figure 14

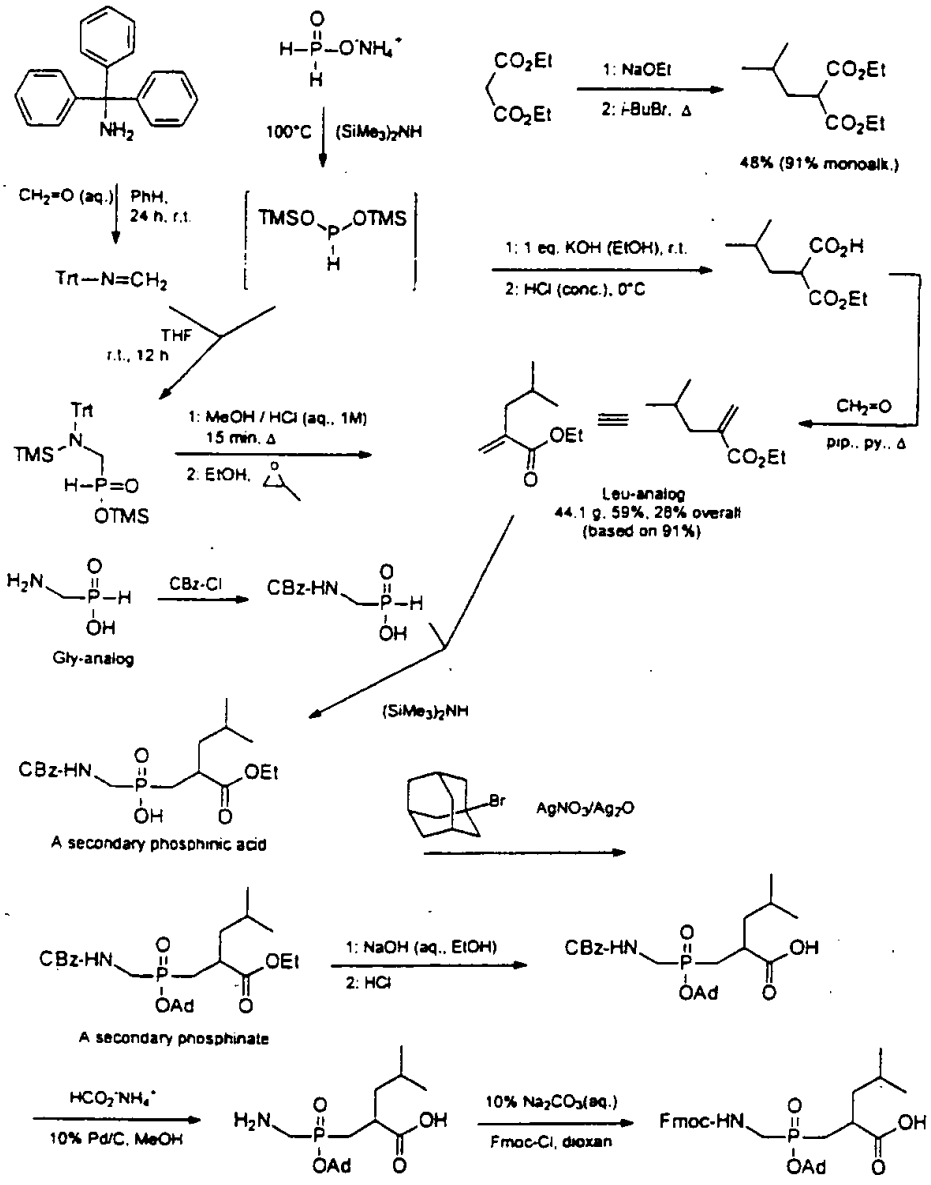
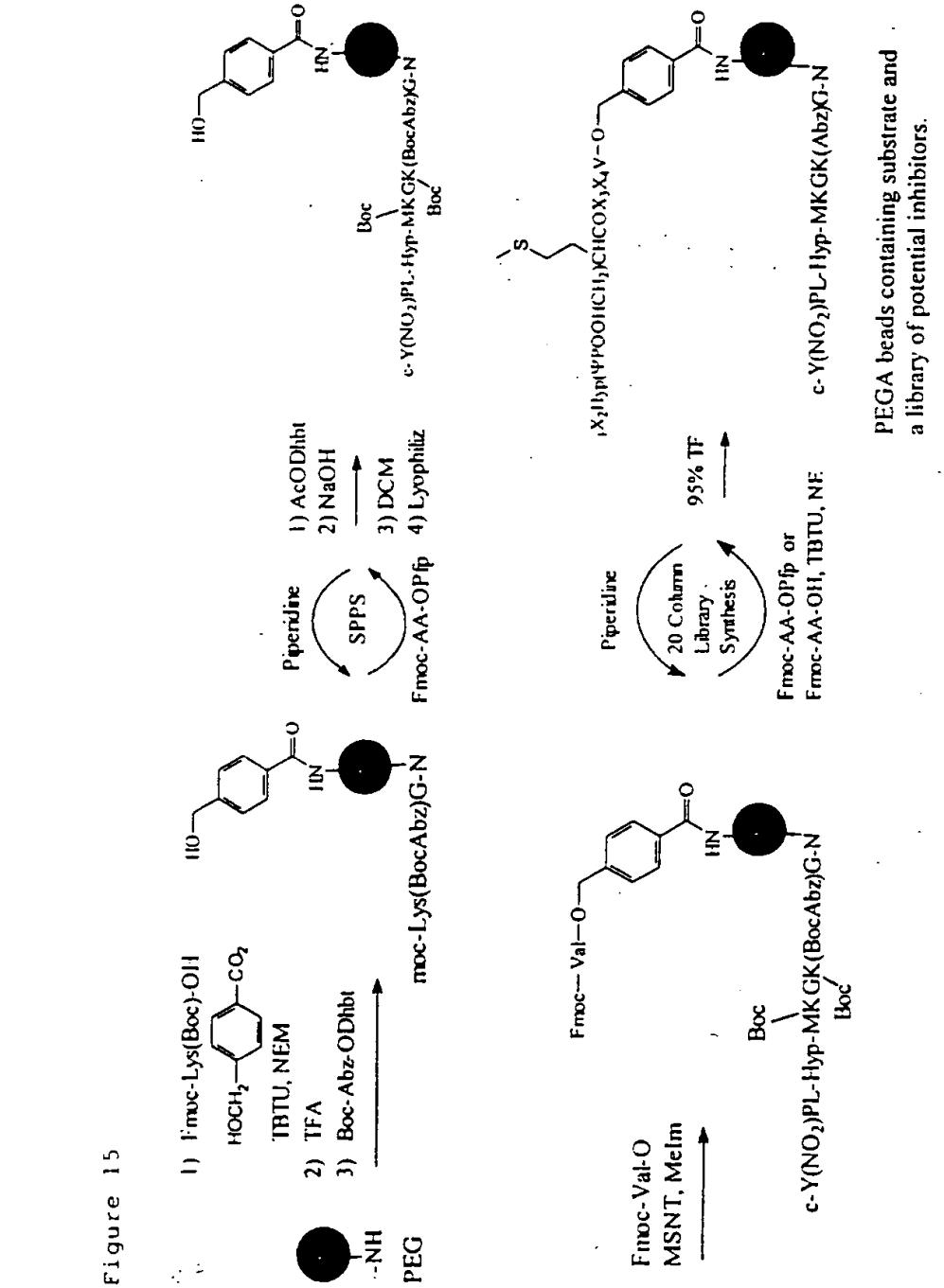

NH2
H–P(=O)(H)–O⁻NH4⁺
100°C
(SiMe3)2NH
CO2Et
CO2Et
1: NaOEt
2: i-BuBr, Δ
CO2Et
CO2Et
48% (91% monoalk.)
CH2=O (aq.)
PhH,
24 h, r.t.
Trt–N=CH2
TMSO
OTMS
P
H
1: 1 eq. KOH (EtOH), r.t.
2: HCl (conc.), 0°C
CO2H
CO2Et
THF
r.t., 12 h
Trt
TMS
N
H–P=O
OTMS
1: MeOH / HCl (aq., 1M)
15 min, Δ
2: EtOH,
OEt
CO2Et
CH2=O
pip., py., Δ
Leu-analog
44.1 g, 59%, 28% overall
(based on 91%)
H2N
P–H
OH
CBz-Cl
CBz-HN
P–H
OH
Gly-analog
(SiMe3)2NH
CBz-HN
OEt
OH
A secondary phosphinic acid
Br
AgNO3/Ag2O
CBz-HN
OEt
OAd
A secondary phosphinate
1: NaOH (aq., EtOH)
2: HCl
CBz-HN
OH
OAd
HCO2⁻NH4⁺
10% Pd/C, MeOH
H2N
OH
OAd
10% Na2CO3(aq.)
Fmoc-Cl, dioxan
Fmoc-HN
OH
OAd

Figure 15


1) Fmoc-Lys(Boc)-OH
TBTU, NEM
2) TFA
3) Boc-Abz-ODhbt
PEG
moc-Lys(BocAbz)G-N
Piperidine
SPPS
Fmoc-AA-OPfp
1) AcODhbt
2) NaOH
3) DCM
4) Lyophiliz
c-Y(NO2)PL-Hyp-MKGK(BocAbz)G-N
Fmoc-Val-O
MSNT, MeIm
Piperidine
20 Column Library Synthesis
Fmoc-AA-OPfp or Fmoc-AA-OH, TBTU, NE
95% TF
PEGA beads containing substrate and a library of potential inhibitors.

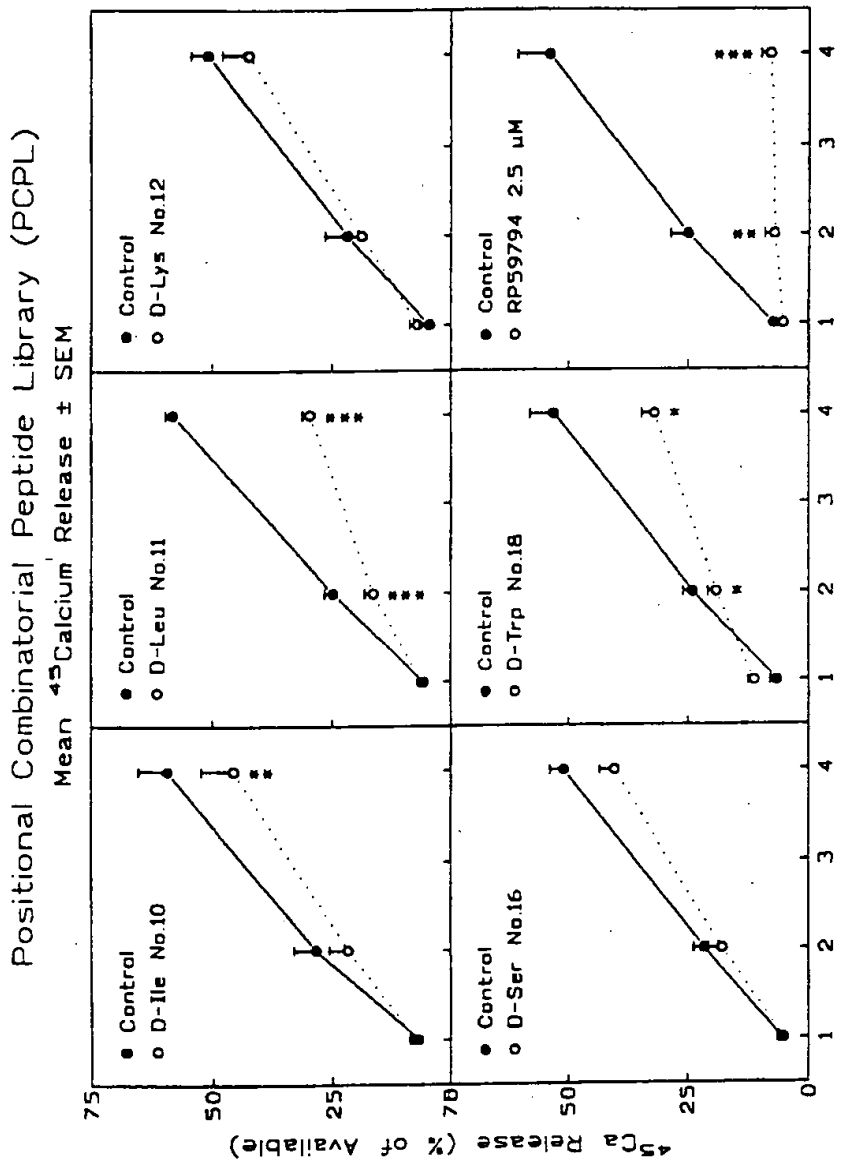

Positional Combinatorial Peptide Library (PCPL)
Mean ^{45}Calcium Release ± SEM
^{45}Ca Release (% of Available)
Time (Days)
Control
D-Ile No.10
D-Leu No.11
D-Lys No.12
D-Ser No.16
D-Trp No.18
RP59794 2.5 µM

Figure 16

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/EP 97/04110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 C07K7/04 C07K14/81 C12N15/52

According to international Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SATO T. ET AL: "Identification of the membrane-type matrix metalloproteinase MT1-MMP in osteoclasts" JOURNAL OF CELL SCIENCE, vol. 110, March 1997, pages 589-596, XP002049983 see the whole document ---	1-12
X	EP 0 611 756 A (TAKEDA CHEMICAL INDUSTRIES LTD) 24 August 1994 cited in the application	10,11
A	see page 2 ---	1-9,12
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1997	16.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Olsen, L

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 97/04110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIRES U.I. ET AL.: "Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin-B" THE JOURNAL OF CLINICAL INVESTIGATION, vol. 95, 1995, pages 2089-2095, XP002049984	10,11
A	see the whole document	1-9,12
X	EVERTS V. ET AL.: "Degradation of collagen in the bone-resorbing compartment underlaying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases" JOURNAL OF CELLULAR PHYSIOLOGY, vol. 150, 1992, pages 221-231, XP002049985	10,11
A	see the whole document	1-9,12
X	BIRKEDAL-HANSEN H. ET AL.: "Matrix metalloproteinases: A review" CRITICAL REVIEWS IN ORAL BIOLOGY AND MEDICINE, vol. 4, 1993, pages 197-250, XP002049986 cited in the application	10,11
A	see the whole document	1-9,12
X	DELAISSE J.-M. AND VAES G.: "Mechanism of mineral solubilization and matrix degradation in osteoclastic bone resorption" 1992 , BIOLOGY AND PHYSIOLOGY OF THE OSTEOCLAST; EDIT.: RIFKIN B.R. AND GAY C.V., CRC PRESS, BOCA RATON, ANN ARBOR, LONDON, TOKYO XP002049988 cited in the application	10,11
A	see the whole document	1-9,12
A	SAKAI D. ET AL.: "Osteoclast molecular phenotyping by random cDNA sequencing" BONE, vol. 17, 1995, pages 111-119, XP002049987 cited in the application see the whole document	1-12
A	SATO H. ET AL.: "A matrix metalloproteinase expressed on the surface of invasive tumour cells" NATURE, vol. 370, no. 6484, 7 July 1994, pages 61-65, XP000578393 cited in the application see the whole document	1-12

-/--

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/EP 97/04110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 685 557 A (FUJI YAKUHIN KOGYO KK) 6 December 1995 cited in the application see the whole document -----	1-12

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/EP 97/04110

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